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<p>(21) International Application Number: PCT/US97/22629  (22) International Filing Date: 4 December 1997 (04.12.97)  (30) Priority Data: 60/033,230 5 December 1996 (05.12.96) US  (71) Applicant: CORNELL RESEARCH FOUNDATION, INC. [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US).  (72) Inventors: QIU, Dwen; Apartment #2-I, 110 Dryden Road, Ithaca, NY 14850 (US). WEI, Zhong-Min; 8230 125th Court, Kirkland, WA 98034 (US). BEER, Steven, V.; 211 Hudson Street, Ithaca, NY 14850 (US).  (74) Agents: GOLDMAN, Michael, L. et al.; Nixon, Hargrave, Devans &amp; Doyle LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: HYPERSENSITIVE RESPONSE INDUCED RESISTANCE IN PLANTS BY SEED TREATMENT</p>		
<p>(57) Abstract</p> <p>The present invention relates to a method of imparting pathogen resistance to plants. This involves applying a hypersensitive response elicitor polypeptide or protein in a non-infectious form to a plant seed under conditions where the polypeptide or protein contacts cells of the plant seed. The present invention is also directed to a pathogen resistance imparting plant seed. Alternatively, transgenic plant seeds containing a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein can be planted in soil and a plant can be propagated from the planted seed under conditions effective to impart pathogen resistance to the plant.</p>		

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HYPERSENSITIVE RESPONSE INDUCED RESISTANCE  
IN PLANTS BY SEED TREATMENT

5           This application claims the benefit of U.S.  
Provisional Patent Application Serial No. 60/033,230,  
filed December 5, 1996.

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U.S. Government under USDA NRI Competitive Research Grant  
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FIELD OF THE INVENTION

          The present invention relates to imparting  
15   hypersensitive response induced resistance to plants by  
treatment of seeds.

BACKGROUND OF THE INVENTION

20           Living organisms have evolved a complex array  
of biochemical pathways that enable them to recognize and  
respond to signals from the environment. These pathways  
include receptor organs, hormones, second messengers, and  
enzymatic modifications. At present, little is known  
25   about the signal transduction pathways that are activated  
during a plant's response to attack by a pathogen,  
although this knowledge is central to an understanding of  
disease susceptibility and resistance. A common form of  
plant resistance is the restriction of pathogen  
30   proliferation to a small zone surrounding the site of  
infection. In many cases, this restriction is  
accompanied by localized death (i.e., necrosis) of host  
tissues. Together, pathogen restriction and local tissue  
necrosis characterize the hypersensitive response. In  
35   addition to local defense responses, many plants respond  
to infection by activating defenses in uninfected parts  
of the plant. As a result, the entire plant is more  
resistant to a secondary infection. This systemic

acquired resistance can persist for several weeks or more (R.E.F. Matthews, Plant Virology (Academic Press, New York, ed. 2, 1981)) and often confers cross-resistance to unrelated pathogens (J. Kuc, in Innovative Approaches to Plant Disease Control, I. Chet, Ed. (Wiley, New York, 1987), pp. 255-274, which is hereby incorporated by reference). See also Kessman, et al., "Induction of Systemic Acquired Disease Resistance in Plants By Chemicals," Ann. Rev. Phytopathol. 32:439-59 (1994), Ryals, et al., "Systemic Acquired Resistance," The Plant Cell 8:1809-19 (Oct. 1996), and Neuenschwander, et al., "Systemic Acquired Resistance," Plant-Microbe Interactions vol. 1, G. Stacey, et al. ed. pp. 81-106 (1996), which are hereby incorporated by reference.

Expression of systemic acquired resistance is associated with the failure of normally virulent pathogens to ingress the immunized tissue (Kuc, J., "Induced Immunity to Plant Disease," Bioscience, 32:854-856 (1982), which is hereby incorporated by reference). Establishment of systemic acquired resistance is correlated with systemic increases in cell wall hydroxyproline levels and peroxidase activity (Smith, J.A., et al., "Comparative Study of Acidic Peroxidases Associated with Induced Resistance in Cucumber, Muskmelon and Watermelon," Physiol. Mol. Plant Pathol. 14:329-338 (1988), which is hereby incorporated by reference) and with the expression of a set of nine families of so-called systemic acquired resistance gene (Ward, E.R., et al., "Coordinate Gene Activity in Response to Agents that Induce Systemic Acquired Resistance," Plant Cell 3:49-59 (1991), which is hereby incorporated by reference). Five of these defense gene families encode pathogenesis-related proteins whose physiological functions have not been established. However, some of these proteins have antifungal activity in vitro (Bol,

- 3 -

J.F., et al., "Plant Pathogenesis-Related Proteins Induced by Virus Infection," Ann. Rev. Phytopathol. 28:113-38 (1990), which is hereby incorporated by reference) and the constitutive expression of a bean chitinase gene in transgenic tobacco protects against infection by the fungus *Rhizoctonia solani* (Broglie, K., et al., "Transgenic Plants with Enhanced Resistance to the Fungal Pathogen *Rhizoctonia Solani*," Science 254:1194-1197 (1991), which is hereby incorporated by reference), suggesting that these systemic acquired resistance proteins may contribute to the immunized state (Uknes, S., et al., "Acquired Resistance in *Arabidopsis*," Plant Cell 4:645-656 (1992), which is hereby incorporated by reference).

Salicylic acid appears to play a signal function in the induction of systemic acquired resistance since endogenous levels increase after immunization (Malamy, J., et al., "Salicylic Acid: A Likely Endogenous Signal in the Resistance Response of Tobacco to Viral Infection," Science 250:1002-1004 (1990), which is hereby incorporated by reference) and exogenous salicylate induces systemic acquired resistance genes (Yalpani, N., et al., "Salicylic Acid is a Systemic Signal and an Inducer of Pathogenesis-Related Proteins in Virus-Infected Tobacco," Plant Cell 3:809-818 (1991), which is hereby incorporated by reference), and acquired resistance (Uknes, S., et al., "Acquired Resistance in *Arabidopsis*," Plant Cell 4:645-656 (1992), which is hereby incorporated by reference). Moreover, transgenic tobacco plants in which salicylate is destroyed by the action of a bacterial transgene encoding salicylate hydroxylase do not exhibit systemic acquired resistance (Gaffney, T., et al., "Requirement of Salicylic Acid for the Induction of Systemic Acquired Resistance," Science 261:754-56 (1993), which is hereby incorporated by

reference). However, this effect may reflect inhibition of a local rather than a systemic signal function, and detailed kinetic analysis of signal transmission in cucumber suggests that salicylate may not be essential for long-distance signaling (Rasmussen, J.B., et al., "Systemic Induction of Salicylic Acid Accumulation in Cucumber after Inoculation with *Pseudomonas Syringae* pv. *Syringae*," Plant Physiol. 97:1342-1347) (1991), which is hereby incorporated by reference).

10 Immunization using biotic agents has been extensively studied. Green beans were systemically immunized against disease caused by cultivar-pathogenic races of *Colletotrichum lindemuthianum* by prior infection with either cultivar-nonpathogenic races (Rahe, J.E.,  
15 "Induced Resistance in *Phaseolus Vulgaris* to Bean Anthracnose," Phytopathology 59:1641-5 (1969); Elliston, J., et al., "Induced Resistance to Anthracnose at a Distance from the Site of the Inducing Interaction," Phytopathology 61:1110-12 (1971); Skipp, R., et al.,  
20 "Studies on Cross Protection in the Anthracnose Disease of Bean," Physiological Plant Pathology 3:299-313 (1973), which are hereby incorporated by reference), cultivar-pathogenic races attenuated by heat in host tissue prior to symptom appearance (Rahe, J.E., et al.,  
25 "Metabolic Nature of the Infection-Limiting Effect of Heat on Bean Anthracnose," Phytopathology 60:1005-9 (1970), which is hereby incorporated by reference) or nonpathogens of bean. The anthracnose pathogen of cucumber, *Colletotrichum lagenarium*, was equally  
30 effective as non-pathogenic races as an inducer of systemic protection against all races of bean anthracnose. Protection was induced by *C. lagenarium* in cultivars resistant to one or more races of *C. lindemuthianum* as well as in cultivars susceptible to all  
35 reported races of the fungus and which accordingly had

been referred to as 'lacking genetic resistance' to the pathogen (Elliston, J., et al., "Protection of Bean Against Anthracnose by *Colletotrichum* Species Nonpathogenic on Bean," Phytopathologische Zeitschrift 86:117-26 (1976); Elliston, J., et al., "A Comparative Study on the Development of Compatible, Incompatible and Induced Incompatible Interactions Between *Collectotrichum* Species and *Phaseolus Vulgaris*," Phytopathologische Zeitschrift 87:289-303 (1976), which are hereby incorporated by reference). These results suggest that the same mechanisms may be induced in cultivars reported as 'possessing' or 'lacking' resistance genes (Elliston, J., et al., "Relation of Phytoalexin Accumulation to Local and Systemic Protection of Bean Against Anthracnose," Phytopathologische Zeitschrift 88:114-30 (1977), which is hereby incorporated by reference). It also is apparent that cultivars susceptible to all races of *C. lindemuthianum* do not lack genes for induction of resistance mechanisms against the pathogen.

Kuc, J., et al., "Protection of Cucumber Against *Collectotrichum Lagenarium* by *Colletotrichum Lagenarium*," Physiological Plant Pathology 7:195-9 (1975), which is hereby incorporated by reference), showed that cucumber plants could be systemically protected against disease caused by *Colletotrichum lagenarium* by prior inoculation of the cotyledons or the first true leaf with the same fungus. Subsequently, cucumbers have been systemically protected against fungal, bacterial, and viral diseases by prior localized infection with either fungi, bacteria, or viruses (Hammerschmidt, R., et al., "Protection of Cucumbers Against *Colletotrichum Lagenarium* and *Cladosporium Cucumerinum*," Phytopathology 66:790-3 (1976); Jenns, A. E., et al., "Localized Infection with Tobacco Necrosis Virus Protects Cucumber Against *Colletotrichum*

*Lagenarium*," Physiological Plant Pathology 11:207-12 (1977); Caruso, F.L., et al. "Induced Resistance of Cucumber to Anthracnose and Angular Leaf Spot by *Pseudomonas Lachrymans* and *Colletotrichum Lagenarium*," Physiological Plant Pathology 14:191-201 (1979); Staub, T., et al., "Systemic Protection of Cucumber Plants Against Disease Caused by *Cladosporium Cucumerinum* and *Colletotrichum Lagenarium* by Prior Localized Infection with Either Fungus," Physiological Plant Pathology, 17:389-93 (1980); Bergstrom, G.C., et al., "Effects of Local Infection of Cucumber by *Colletotrichum Lagenarium*, *Pseudomonas Lachrymans* or Tobacco Necrosis Virus on Systemic Resistance to Cucumber Mosaic Virus," Phytopathology 72:922-6 (1982); Gessler, C., et al., "Induction of Resistance to *Fusarium* Wilt in Cucumber by Root and Foliar Pathogens," Phytopathology 72:1439-41 (1982); Basham, B., et al., "Tobacco Necrosis Virus Induces Systemic Resistance in Cucumbers Against *Sphaerotheca Fuliginea*," Physiological Plant Pathology 23:137-44 (1983), which are hereby incorporated by reference). Non-specific protection induced by infection with *C. lagenarium* or tobacco necrosis virus was effective against at least 13 pathogens, including obligatory and facultative parasitic fungi, local lesion and systemic viruses, wilt fungi, and bacteria. Similarly, protection was induced by and was also effective against root pathogens. Other cucurbits, including watermelon and muskmelon have been systemically protected against *C. lagenarium* (Caruso, F.L., et al., "Protection of Watermelon and Muskmelon Against *Colletotrichum Lagenarium* by *Colletotrichum Lagenarium*," Phytopathology 67:1285-9 (1977), which is hereby incorporated by reference).

Systemic protection in tobacco has also been induced against a wide variety of diseases (Kuc, J., et



- 7 -

al., "Immunization for Disease Resistance in Tobacco," Recent Advances in Tobacco Science 9:179-213 (1983), which is hereby incorporated by reference). Necrotic lesions caused by tobacco mosaic virus enhanced

5 resistance in the upper leaves to disease caused by the virus (Ross, A.F., et al., "Systemic Acquired Resistance Induced by Localized Virus Infections in Plants," Virology 14:340-58 (1961); Ross, A.F., et al., "Systemic Effects of Local Lesion Formation," In: Viruses of Plants

10 pp. 127-50 (1966), which are hereby incorporated by reference). *Phytophthora parasitica* var. *nicotianae*, *P. tabacina* and *Pseudomonas tabaci* and reduced reproduction of the aphid *Myzus persicae* (McIntyre, J.L., et al., "Induction of Localized and Systemic Protection Against

15 *Phytophthora Parasitica* var. *nicotianae* by Tobacco Mosaic Virus Infection of Tobacco Hypersensitive to the Virus," Physiological Plant Pathology 15:321-30 (1979); McIntyre, J.L., et al., "Effects of Localized Infections of *Nicotiana Tabacum* by Tobacco Mosaic Virus on Systemic

20 Resistance Against Diverse Pathogens and an Insect," Phytopathology 71:297-301 (1981), which are hereby incorporated by reference). Infiltration of heat-killed *Pseudomonas tabacin* (Lovrekovich, L., et al., "Induced Reaction Against Wildfire Disease in Tobacco Leaves

25 Treated with Heat-Killed Bacteria," Nature 205:823-4 (1965), which is hereby incorporated by reference), and *Pseudomonas solanacearum* (Sequeira, L, et al., "Interaction of Bacteria and Host Cell Walls: Its Relation to Mechanisms of Induced Resistance,"

30 Physiological Plant Pathology 10:43-50 (1977), which is hereby incorporated by reference), into tobacco leaves induced resistance against the same bacteria used for infiltration. Tobacco plants were also protected by the nematode *Pratylenchus penetrans* against *P. parasitica*

35 var. *nicotiana* (McIntyre, J.L., et al. "Protection of

Tobacco Against *Phytophthora Parasitica* Var. *Nicotianae* by Cultivar-Nonpathogenic Races, Cell-Free Sonicates and *Pratylenchus Penetrans*," Phytopathology 68:235-9 (1978), which is hereby incorporated by reference).

- 5           Cruikshank, I.A.M., et al., "The Effect of Stem Infestation of Tobacco with *Peronospora Tabacina* Adam on Foliage Reaction to Blue Mould," Journal of the Australian Institute of Agricultural Science 26:369-72 (1960), which is hereby incorporated by reference, were
- 10 the first to report immunization of tobacco foliage against blue mould (i.e., *P. tabacina*) by stem injection with the fungus, which also resulted in dwarfing and premature senescence. It was recently discovered that injection external to the xylem not only alleviated
- 15 stunting but also promoted growth and development. Immunized tobacco plants, in both glasshouse and field experiments, were approximately 40% taller, had a 40% increase in dry weight, a 30% increase in fresh weight, and 4-6 more leaves than control plants (Tuzun, S., et
- 20 al., "The Effect of Stem Injections with *Peronospora Tabacina* and Metalaxyl Treatment on Growth of Tobacco and Protection Against Blue Mould in the Field," Phytopathology 74:804 (1984), which is hereby incorporated by reference). These plants flowered
- 25 approximately 2-3 weeks earlier than control plants (Tuzun, S., et al., "Movement of a Factor in Tobacco Infected with *Peronospora Tabacina* Adam which Systemically Protects Against Blue Mould," Physiological Plant Pathology 26:321-30 (1985), which is hereby
- 30 incorporated by reference).

Systemic protection does not confer absolute immunity against infection, but reduces the severity of the disease and delays symptom development. Lesion number, lesion size, and extent of sporulation of fungal

- 9 -

pathogens are all decreased. The diseased area may be reduced by more than 90%.

When cucumbers were given a 'booster' inoculation 3-6 weeks after the initial inoculation, immunization induced by *C. lagenarium* lasted through flowering and fruiting (Kuc, J., et al., "Aspects of the Protection of Cucumber Against *Colletotrichum Lagenarium* by *Colletotrichum Lagenarium*," Phytopathology 67:533-6 (1977), which is hereby incorporated by reference).

Protection could not be induced once plants had set fruit. Tobacco plants were immunized for the growing season by stem injection with sporangia of *P. tabacina*. However, to prevent systemic blue mould development, this technique was only effective when the plants were above 20 cm in height.

Removal of the inducer leaf from immunized cucumber plants did not reduce the level of immunization of pre-existing expanded leaves. However, leaves which subsequently emerged from the apical bud were progressively less protected than their predecessors (Dean, R.A., et al., "Induced Systemic Protection in Cucumber: Time of Production and Movement of the 'Signal'," Phytopathology 76:966-70 (1986), which is hereby incorporated by reference). Similar results were reported by Ross, A.F., "Systemic Effects of Local Lesion Formation," In: Viruses of Plants pp. 127-50 (1966), which is hereby incorporated by reference, with tobacco (local lesion host) immunized against tobacco mosaic virus by prior infection with tobacco mosaic virus. In contrast, new leaves which emerged from scions excised from tobacco plants immunized by stem-injection with *P. tabacina* were highly protected (Tuzun, S., et al., "Transfer of Induced Resistance in Tobacco to Blue Mould (*Peronospora tabacina* Adam.) Via Callus," Phytopathology 75:1304 (1985), which is hereby incorporated by

reference). Plants regenerated via tissue culture from leaves of immunized plants showed a significant reduction in blue mould compared to plants regenerated from leaves of non-immunized parents. Young regenerants only showed reduced sporulation. As plants aged, both lesion development and sporulation were reduced. Other investigators, however, did not reach the same conclusion, although a significant reduction in sporulation in one experiment was reported (Lucas, J.A., et al., "Nontransmissibility to Regenerants from Protected Tobacco Explants of Induced Resistance to *Peronospora Hyoscyami*," Phytopathology 75:1222-5 (1985), which is hereby incorporated by reference).

Protection of cucumber and watermelon is effective in the glasshouse and in the field (Caruso, F.L., et al., "Field Protection of Cucumber Against *Colletotrichum Lagenarium* by *C. Lagenarium*," Phytopathology 67:1290-2 (1977), which is hereby incorporated by reference). In one trial, the total lesion area of *C. lagenarium* on protected cucumber was less than 2% of the lesion areas on unprotected control plants. Similarly, only 1 of 66 protected, challenged plants died, whereas 47 of 69 unprotected, challenged watermelons died. In extensive field trials in Kentucky and Puerto Rico, stem injection of tobacco with sporangia of *P. tabacina* was at least as effective in controlling blue mould as the best fungicide, metalaxyl. Plants were protected, leading to a yield increase of 10-25% in cured tobacco.

Induced resistance against bacteria and viruses appears to be expressed as suppression of disease symptoms or pathogen multiplication or both (Caruso, F.L., et al., "Induced Resistance of Cucumber to Anthracnose and Angular Leaf Spot by *Pseudomonas Lachrymans* and *Colletotrichum Lagenarium*," Physiological

- 11 -

Plant Pathology 14:191-201 (1979); Doss, M., et al.,  
"Systemic Acquired Resistance of Cucumber to *Pseudomonas*  
*Lachrymans* as Expressed in Suppression of Symptoms, but  
not in Multiplication of Bacteria," Acta Phytopathologia  
5 Academiae Scientiarum Hungaricae 16:(3-4), 269-72 (1981);  
Jenns, A.E., et al., "Non-Specific Resistance to  
Pathogens Induced Systemically by Local Infection of  
Cucumber with Tobacco Necrosis Virus, *Colletotrichum*  
*Lagenarium* or *Pseudomonas Lachrymans*," Phytopathologia  
10 Mediterranea 18:129-34 (1979), which are hereby  
incorporated by reference).

As described above, research concerning  
systemic acquired resistance involves infecting plants  
with infectious pathogens. Although studies in this area  
15 are useful in understanding how systemic acquired  
resistance works, eliciting such resistance with  
infectious agents is not commercially useful, because  
such plant-pathogen contact can weaken or kill plants.  
The present invention is directed to overcoming this  
20 deficiency.

#### SUMMARY OF THE INVENTION

The present invention relates to a method of  
25 producing plant seeds which impart pathogen resistance to  
plants grown from the seeds. This method involves  
applying a hypersensitive response elicitor polypeptide  
or protein in a non-infectious form to plant seeds under  
conditions where the polypeptide or protein contacts  
30 cells of the plant seeds.

As an alternative to applying a hypersensitive  
response elicitor polypeptide or protein to plant seeds  
in order to impart pathogen resistance to plants grown  
from the seeds, transgenic seeds can be utilized. This  
35 involves providing a transgenic plant seed transformed

with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein and planting that seed in soil. A plant is then propagated from the planted seed under conditions effective to impart pathogen resistance to the plant.

Another aspect of the present invention relates to a pathogen-resistance imparting plant seed to which a non-infectious hypersensitive response elicitor polypeptide or protein has been applied.

The present invention has the potential to: treat plant diseases which were previously untreatable; treat diseases systemically that one would not want to treat separately due to cost; and avoid the use of agents that have an unpredictable effect on the environment and even the plants. The present invention can impart resistance without using agents which are harmful to the environment or pathogenic to the plant seeds being treated or to plants situated near the location that treated seeds are planted. Since the present invention involves use of a natural product that is fully and rapidly biodegradable, the environment would not be contaminated.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of producing plant seeds which impart pathogen resistance to plants grown from the seeds. This method involves applying a hypersensitive response elicitor polypeptide or protein in a non-infectious form to a plant seed under conditions effective to impart disease resistance to a plant grown from the seed.

As an alternative to applying a hypersensitive response elicitor polypeptide or protein to plant seeds in order to impart pathogen resistance to plants grown

from the seeds, transgenic seeds can be utilized. This involves providing a transgenic plant seed transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein and planting that seed in soil. A plant is then propagated from the planted seed under conditions effective to impart pathogen resistance to the plant.

Another aspect of the present invention relates to a pathogen-resistance imparting plant seed to which a non-infectious hypersensitive response elicitor polypeptide or protein has been applied.

The hypersensitive response elicitor polypeptide or protein utilized in the present invention can correspond to hypersensitive response elicitor polypeptides or proteins derived from a wide variety of fungal and bacterial pathogens. Such polypeptides or proteins are able to elicit local necrosis in plant tissue contacted by the elicitor.

Examples of suitable bacterial sources of polypeptide or protein elicitors include *Erwinia*, *Pseudomonas*, and *Xanthomonas* species (e.g., the following bacteria: *Erwinia amylovora*, *Erwinia chrysanthemi*, *Erwinia stewartii*, *Erwinia carotovora*, *Pseudomonas syringae*, *Pseudomonas solanacearum*, *Xanthomonas campestris*, or mixtures thereof).

An example of a fungal source of a hypersensitive response elicitor protein or polypeptide is *Phytophthora*. Suitable species of such fungal pathogens include *Phytophthora parasitica*, *Phytophthora cryptogea*, *Phytophthora cinnamomi*, *Phytophthora capsici*, *Phytophthora megasperma*, and *Phytophthora citrophthora*.

The embodiment of the present invention where the hypersensitive response elicitor polypeptide or protein is applied to the plant seed can be carried out

- 14 -

in a number of ways, including: 1) application of an isolated elicitor polypeptide or protein; 2) application of bacteria which do not cause disease and are transformed with genes encoding a hypersensitive response elicitor polypeptide or protein; and 3) application of bacteria which cause disease in some plant species (but not in those to which they are applied) and naturally contain a gene encoding the hypersensitive response elicitor polypeptide or protein. In addition, seeds in

10 accordance with the present invention can be recovered from plants which have been treated with a hypersensitive response elicitor protein or polypeptide in accordance with the present invention.

In one embodiment of the present invention, 15 the hypersensitive response elicitor polypeptides or proteins to be applied can be isolated from their corresponding organisms and applied to plants. Such isolation procedures are well known, as described in Arlat, M., F. Van Gijsegem, J. C. Huet, J. C. Pemollet, 20 and C. A. Boucher, "PopA1, a Protein which Induces a Hypersensitive-like Response in Specific *Petunia* Genotypes is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543 - 553 (1994); He, S. Y., H. C. Huang, and A. Collmer, "*Pseudomonas syringae* pv. 25 *syringae* Harpin<sub>PSS</sub>: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993); and Wei, Z.-M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S.-Y. He, A. Collmer, and S. V. Beer, "Harpin Elicitor of the Hypersensitive 30 Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which are hereby incorporated by reference. See also pending U.S. Patent Application Serial Nos. 08/200,024 and 08/062,024, which are hereby incorporated by reference. Preferably, 35 however, the isolated hypersensitive response elicitor



- 15 -

polypeptides or proteins of the present invention are produced recombinantly and purified as described below.

In other embodiments of the present invention, the hypersensitive response elicitor polypeptide or protein of the present invention can be applied to plant seeds by applying bacteria containing genes encoding the hypersensitive response elicitor polypeptide or protein. Such bacteria must be capable of secreting or exporting the polypeptide or protein so that the elicitor can contact plant seed cells. In these embodiments, the hypersensitive response elicitor polypeptide or protein is produced by the bacteria after application to the seeds or just prior to introduction of the bacteria to the seeds.

In one embodiment of the bacterial application mode of the present invention, the bacteria to be applied do not cause the disease and have been transformed (e.g., recombinantly) with genes encoding a hypersensitive response elicitor polypeptide or protein. For example, *E. coli*, which do not elicit a hypersensitive response in plants, can be transformed with genes encoding a hypersensitive response elicitor polypeptide and other related proteins required for production and secretion of the elicitor which is then applied to plant seeds. Expression of this polypeptide or protein can then be caused to occur. Bacterial species (other than *E. coli*) can also be used in this embodiment of the present invention.

In another embodiment of the bacterial application mode of the present invention, the bacteria do cause disease and naturally contain a gene encoding a hypersensitive response elicitor polypeptide or protein. Examples of such bacteria are noted above. However, in this embodiment these bacteria are applied to plant seeds for plants which are not susceptible to the disease

- 16 -

carried by the bacteria. For example, *Erwinia amylovora* causes disease in apple or pear but not in tomato. However, such bacteria will elicit a hypersensitive response in tomato. Accordingly, in accordance with this embodiment of the present invention, *Erwinia amylovora* can be applied to tomato seeds to impart pathogen resistance without causing disease in plants of that species.

#### The hypersensitive response-elicitor

10 polypeptide or protein from *Erwinia chrysanthemi* has an amino acid sequence corresponding to SEQ. ID. No. 1 as follows:

15	Met	Gln	Ile	Thr	Ile	Lys	Ala	His	Ile	Gly	Gly	Asp	Leu	Gly	Val	Ser	1	5	10	15
	Gly	Leu	Gly	Ala	Gln	Gly	Leu	Lys	Gly	Leu	Asn	Ser	Ala	Ala	Ser	Ser	20	25	30	
20	Leu	Gly	Ser	Ser	Val	Asp	Lys	Leu	Ser	Ser	Thr	Ile	Asp	Lys	Leu	Thr	35	40	45	
	Ser	Ala	Leu	Thr	Ser	Met	Met	Phe	Gly	Gly	Ala	Leu	Ala	Gln	Gly	Leu	50	55	60	
25	Gly	Ala	Ser	Ser	Lys	Gly	Leu	Gly	Met	Ser	Asn	Gln	Leu	Gly	Gln	Ser	65	70	75	80
30	Phe	Gly	Asn	Gly	Ala	Gln	Gly	Ala	Ser	Asn	Leu	Leu	Ser	Val	Pro	Lys	85	90	95	
	Ser	Gly	Gly	Asp	Ala	Leu	Ser	Lys	Met	Phe	Asp	Lys	Ala	Leu	Asp	Asp	100	105	110	
35	Leu	Leu	Gly	His	Asp	Thr	Val	Thr	Lys	Leu	Thr	Asn	Gln	Ser	Asn	Gln	115	120	125	
	Leu	Ala	Asn	Ser	Met	Leu	Asn	Ala	Ser	Gln	Met	Thr	Gln	Gly	Asn	Met	130	135	140	
40	Asn	Ala	Phe	Gly	Ser	Gly	Val	Asn	Asn	Ala	Leu	Ser	Ser	Ile	Leu	Gly	145	150	155	160
45	Asn	Gly	Leu	Gly	Gln	Ser	Met	Ser	Gly	Phe	Ser	Gln	Pro	Ser	Leu	Gly	165	170	175	
	Ala	Gly	Gly	Leu	Gln	Gly	Leu	Ser	Gly	Ala	Gly	Ala	Phe	Asn	Gln	Leu	180	185	190	
50	Gly	Asn	Ala	Ile	Gly	Met	Gly	Val	Gly	Gln	Asn	Ala	Ala	Leu	Ser	Ala	195	200	205	

- 17 -

Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val  
 210 215 220  
 5 Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp  
 225 230 235 240  
 Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp  
 245 250 255  
 10 Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys  
 260 265 270  
 Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln  
 275 280 285  
 15 Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr  
 290 295 300  
 20 Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala  
 305 310 315 320  
 Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala  
 325 330 335  
 25 Asn Ala

This hypersensitive response elicitor polypeptide or  
 protein has a molecular weight of 34 kDa, is heat stable,  
 30 has a glycine content of greater than 16%, and contains  
 substantially no cysteine. The *Erwinia chrysanthemi*  
 hypersensitive response elicitor polypeptide or protein  
 is encoded by a DNA molecule having a nucleotide sequence  
 corresponding to SEQ. ID. No. 2 as follows:

35 CGATTTTACC CGGGTGAACG TGCTATGACC GACAGCATCA CGGTATTCGA CACCGTTACG 60  
 GCGTTTATGG CCGCGATGAA CCGGCATCAG GCGGCGCGCT GGTGCGCCGA ATCCGGCGTC 120  
 40 GATCTGGTAT TTCAGTTTGG GGACACCGGG CGTGAACTCA TGATGCAGAT TCAGCCGGGG 180  
 CAGCAATATC CCGGCATGTT GCGCACGCTG CTCGCTCGTC GTTATCAGCA GGCGGCAGAG 240  
 TGCGATGGCT GCCATCTGTG CCTGAACGGC AGCGATGTAT TGATCCTCTG GTGGCCGCTG 300  
 45 CCGTCGGATC CCGGCAGTTA TCCGCAGGTG ATCGAACGTT TGTTTGAAC TGGCGGAATG 360  
 ACGTTGCCGT CGCTATCCAT AGCACCGACG GCGCGTCCGC AGACAGGGAA CGGACGCGCC 420  
 50 CGATCATTA GATAAAGGCG GCTTTTTTTT TTGCAAAACG GTAACGGTGA GGAACCGTTT 480  
 CACCGTCGGC GTCACCTAGT AACAAGTATC CATCATGATG CCTACATCGG GATCGGCGTG 540  
 55 GGCATCCGTT GCAGATACTT TTGCGAACAC CTGACATGAA TGAGGAAACG AAATTATGCA 600  
 AATTACGATC AAAGCGCACA TCGGCGGTGA TTTGGGCGTC TCCGGTCTGG GGCTGGGTGC 660

- 18 -

	TCAGGGACTG	AAAGGACTGA	ATTCCGCGGC	TTCATCGCTG	GGTTCCAGCG	TGGATAAACT	720
	GAGCAGCACC	ATCGATAAGT	TGACCTCCGC	GCTGACTTCG	ATGATGTTTG	GCGGCGCGCT	780
5	GGCGCAGGGG	CTGGGCGCCA	GCTCGAAGGG	GCTGGGGATG	AGCAATCAAC	TGGGCCAGTC	840
	TTTCGGCAAT	GGCGCGCAGG	GTGCGAGCAA	CCTGCTATCC	GTACCGAAAT	CCGGCGGCGA	900
10	TGCGTTGTCA	AAAATGTTTG	ATAAAGCGCT	GGACGATCTG	CTGGGTCATG	ACACCGTGAC	960
	CAAGCTGACT	AACCAGAGCA	ACCAACTGGC	TAATTCAATG	CTGAACGCCA	GCCAGATGAC	1020
	CCAGGGTAAT	ATGAATGCGT	TCGGCAGCGG	TGTGAACAAC	GCACTGTCGT	CCATTCTCGG	1080
15	CAACGGTCTC	GGCCAGTCGA	TGAGTGGCTT	CTCTCAGCCT	TCTCTGGGGG	CAGGCGGCTT	1140
<hr/>							
	GCAGGGCCTG	AGCGGCGCGG	GTGCATTCAA	CCAGTTGGGT	AATGCCATCG	GCATGGGCGT	1200
20	GGGGCAGAAT	GCTGCGCTGA	GTGCGTTGAG	TAACGTCAGC	ACCCACGTAG	ACGGTAACAA	1260
	CCGCCACTTT	GTAGATAAAG	AAGATCGCGG	CATGGCGAAA	GAGATCGGCC	AGTTTATGGA	1320
	TCAGTATCCG	GAAATATTCG	GTAAACCGGA	ATACCAGAAA	GATGGCTGGA	GTTCCCGGAA	1380
25	GACGGACGAC	AAATCCTGGG	CTAAAGCGCT	GAGTAAACCG	GATGATGACG	GTATGACCGG	1440
	CGCCAGCATG	GACAAATTCC	GTCAGGCGAT	GGGTATGATC	AAAAGCGCGG	TGGCGGGTGA	1500
30	TACCGGCAAT	ACCAACCTGA	ACCTGCGTGG	CGCGGGCGGT	GCATCGCTGG	GTATCGATGC	1560
	GGCTGTGCTC	GGCGATAAAA	TAGCCAACAT	GTCGCTGGGT	AAGCTGGCCA	ACGCCTGATA	1620
	ATCTGTGCTG	GCCTGATAAA	GCGGAAACGA	AAAAAGAGAC	GGGGAAGCCT	GTCTCTTTTC	1680
35	TTATTATGCG	GTTTATGCGG	TTACCTGGAC	CGGTTAATCA	TCGTCATCGA	TCTGGTACAA	1740
	ACGCACATTT	TCCC GTTCAT	TCGCGTCGTT	ACGCGCCACA	ATCGCGATGG	CATCTTCCTC	1800
40	GTCGCTCAGA	TTGCGCGGCT	GATGGGGAAC	GCCGGGTGGA	ATATAGAGAA	ACTCGCCGGC	1860
	CAGATGGAGA	CACGTCTGCG	ATAAATCTGT	GCCGTAACGT	GTTTCTATCC	GCCCCCTTAG	1920
	CAGATAGATT	GCGGTTTCGT	AATCAACATG	GTAATGCGGT	TCCGCCTGTG	CGCCGGCCGG	1980
45	GATCACCACA	ATATTCATAG	AAAGCTGTCT	TGCACCTACC	GTATCGCGGG	AGATACCGAC	2040
	AAAATAGGGC	AGTTTTTGCG	TGGTATCCGT	GGGGTGTTCC	GGCCTGACAA	TCTTGAGTTG	2100
50	GTTCGTCATC	ATCTTTCTCC	ATCTGGGCGA	CCTGATCGGT	T		2141

The hypersensitive response elicitor polypeptide or protein derived from *Erwinia amylovora* has an amino acid sequence corresponding to SEQ. ID. No. 3 as follows:

55

Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser  
 1 5 10 15

- 19 -

Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln  
 20 25 30  
 5 Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn  
 35 40 45  
 Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met  
 50 55 60  
 10 Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu  
 65 70 75 80  
 Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu  
 85 90 95  
 15 Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr  
 100 105 110  
 20 Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro  
 115 120 125  
 Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser  
 130 135 140  
 25 Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln  
 145 150 155 160  
 Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly  
 165 170 175  
 30 Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu  
 180 185 190  
 35 Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly  
 195 200 205  
 Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly  
 210 215 220  
 40 Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu  
 225 230 235 240  
 Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln  
 245 250 255  
 45 Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln  
 260 265 270  
 50 Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe  
 275 280 285  
 Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met  
 290 295 300  
 55 Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro  
 305 310 315 320  
 Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser  
 325 330 335  
 60 Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn  
 340 345 350

- 20 -

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn  
                   355                                  360                                  365  
 5 Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp  
                   370                                  375                                  380  
 Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu  
                   385                                  390                                  395                                  400  
 10 Gly Ala Ala

This hypersensitive response elicitor polypeptide or  
 15 protein has a molecular weight of about 39 kDa, it has a  
 pI of approximately 4.3, and is heat stable at 100°C for  
 at least 10 minutes. This hypersensitive response  
 elicitor polypeptide or protein has substantially no  
 cysteine. The hypersensitive response elicitor  
 20 polypeptide or protein derived from *Erwinia amylovora* is  
 more fully described in Wei, Z.-M., R. J. Laby, C. H.  
 Zumoff, D. W. Bauer, S.-Y. He, A. Collmer, and S. V.  
 Beer, "Harpin, Elicitor of the Hypersensitive Response  
 Produced by the Plant Pathogen *Erwinia amylovora*,"  
 25 Science 257:85-88 (1992), which is hereby incorporated by  
 reference. The DNA molecule encoding this polypeptide or  
 protein has a nucleotide sequence corresponding to SEQ.  
 ID. No. 4 as follows:

30	AAGCTTCGGC ATGGCACGTT TGACCGTTGG GTCGGCAGGG TACGTTTGAA TTATTCATAA	60
	GAGGAATACG TTATGAGTCT GAATACAAGT GGGCTGGGAG CGTCAACGAT GCAAATTTCT	120
	ATCGGCGGTG CGGGCGGAAA TAACGGGTTG CTGGGTACCA GTCGCCAGAA TGCTGGGTTG	180
35	GGTGGCAATT CTGCACTGGG GCTGGGCGGC GGTAATCAAA ATGATACCGT CAATCAGCTG	240
	GCTGGCTTAC TCACCGGCAT GATGATGATG ATGAGCATGA TGGGCGGTGG TGGGCTGATG	300
40	GGCGGTGGCT TAGGCGGTGG CTTAGGTAAT GGCTTGGGTG GCTCAGGTGG CCTGGGCGAA	360
	GGA CTGTCGA ACGCGCTGAA CGATATGTTA GGCGGTTTCG TGAACACGCT GGGCTCGAAA	420
	GGCGGCAACA ATACCACTTC AACAACAAAT TCCCCGCTGG ACCAGGCGCT GGGTATTAAC	480
45	TCAACGTCCC AAAACGACGA TTCCACCTCC GGCACAGATT CCACCTCAGA CTCCAGCGAC	540
	CCGATGCAGC AGCTGCTGAA GATGTTTCAGC GAGATAATGC AAAGCCTGTT TGGTGATGGG	600
50	CAAGATGGCA CCCAGGGCAG TTCCTCTGGG GGCAAGCAGC CGACCGAAGG CGAGCAGAAC	660

- 21 -

	GCCTATAAAA AAGGAGTCAC TGATGCGCTG TCGGGCCTGA TGGGTAATGG TCTGAGCCAG	720
	CTCCTTGGCA ACGGGGGACT GGGAGGTGGT CAGGGCGGTA ATGCTGGCAC GGGTCTTGAC	780
5	GGTTCGTCGC TGGGCGGCAA AGGGCTGCAA AACCTGAGCG GGCCGGTGGA CTACCAGCAG	840
	TTAGGTAACG CCGTGGGTAC CGGTATCGGT ATGAAAGCGG GCATTTCAGGC GCTGAATGAT	900
10	ATCGGTACGC ACAGGCACAG TTCAACCCGT TCTTTCGTCA ATAAAGGCGA TCGGGCGATG	960
	GCGAAGGAAA TCGGTCAGTT CATGGACCAG TATCCTGAGG TGT TTGGCAA GCCGCAGTAC	1020
	CAGAAAGGCC CGGGTCAGGA GGTGAAAACC GATGACAAAT CATGGGCAAA AGCACTGAGC	1080
15	AAGCCAGATG ACGACGGAAT GACACCAGCC AGTATGGAGC AGTTCAACAA AGCCAAGGGC	1140
	ATGATCAAAA GGCCCATGGC GGGTGATACC GGCAACGGCA ACCTGCAGGC ACGCGGTGCC	1200
20	GGTGGTTCTT CGCTGGGTAT TGATGCCATG ATGGCCGGTG ATGCCATTAA CAATATGGCA	1260
	CTTGGCAAGC TGGGCGCGGC TTAAGCTT	1288

The hypersensitive response elicitor  
 25 polypeptide or protein derived from *Pseudomonas syringae*  
 has an amino acid sequence corresponding to SEQ. ID.  
 No. 5 as follows:

30	Met Gln Ser Leu Ser Leu Asn Ser Ser Ser Leu Gln Thr Pro Ala Met	1 5 10 15
	Ala Leu Val Leu Val Arg Pro Glu Ala Glu Thr Thr Gly Ser Thr Ser	20 25 30
35	Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu Met	35 40 45
	Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala	50 55 60
40	Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp Val	65 70 75 80
45	Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe	85 90 95
	Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met	100 105 110
50	Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu	115 120 125
	Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met	130 135 140
55	Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe Pro	145 150 155 160

- 22 -

Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn Phe  
 165 170 175  
 5 Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile Ile  
 180 185 190  
 Gly Gln Gln Leu Gly Asn Gln Gln Ser Asp Ala Gly Ser Leu Ala Gly  
 195 200 205  
 10 Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser Ser  
 210 215 220  
 Val Met Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp Ser  
 225 230 235 240  
 15 Gly Asn Thr Arg Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile Asp  
 245 250 255  
 20 Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val  
 260 265 270  
 Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln  
 275 280 285  
 25 Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala  
 290 295 300  
 30 Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala  
 305 310 315 320  
 Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg  
 325 330 335  
 35 Asn Gln Ala Ala Ala  
 340

This hypersensitive response elicitor polypeptide or  
 protein has a molecular weight of 34-35 kDa. It is rich  
 40 in glycine (about 13.5%) and lacks cysteine and tyrosine.  
 Further information about the hypersensitive response  
 elicitor derived from *Pseudomonas syringae* is found in  
 He, S. Y., H. C. Huang, and A. Collmer, "*Pseudomonas*  
*syringae* pv. *syringae* Harpin<sub>PSS</sub>: a Protein that is  
 45 Secreted via the Hrp Pathway and Elicits the  
 Hypersensitive Response in Plants," Cell 73:1255-1266  
 (1993), which is hereby incorporated by reference. The  
 DNA molecule encoding the hypersensitive response  
 elicitor from *Pseudomonas syringae* has a nucleotide  
 50 sequence corresponding to SEQ. ID. No. 6 as follows:



- 23 -

ATGCAGAGTC TCAGTCTTAA CAGCAGCTCG CTGCAAACCC CGGCAATGGC CCTTGTCTCTG 60  
 GTACGTCCTG AAGCCGAGAC GACTGGCAGT ACGTCGAGCA AGGCGCTTCA GGAAGTTGTC 120  
 5 GTGAAGCTGG CCGAGGAACT GATGCGCAAT GGTCAACTCG ACGACAGCTC GCCATTGGGA 180  
 AAAGTGTGG CCAAGTCGAT GGCCGCAGAT GGCAAGGCGG GCGGCGGTAT TGAGGATGTC 240  
 10 ATCGCTGCGC TGGACAAGCT GATCCATGAA AAGCTCGGTG ACAACTTCGG CGCGTCTGCG 300  
 GACAGCGCCT CGGGTACCGG ACAGCAGGAC CTGATGACTC AGGTGCTCAA TGGCCTGGCC 360  
 AAGTCGATGC TCGATGATCT TCTGACCAAG CAGGATGGCG GGACAAGCTT CTCCGAAGAC 420  
 15 GATATGCCGA TGCTGAACAA GATCGCGCAG TTCATGGATG ACAATCCCGC ACAGTTTCCC 480  
 AAGCCGGACT CGGGCTCCTG GGTGAACGAA CTCAAGGAAG ACAACTTCCT TGATGGCGAC 540  
 20 GAAACGGCTG CGTTCCGTTT GGCACGCGAC ATCATTGGCC AGCAACTGGG TAATCAGCAG 600  
 AGTGACGCTG GCAGTCTGGC AGGGACGGGT GGAGGTCTGG GCACTCCGAG CAGTTTTTCC 660  
 AACAACTCGT CCGTGATGGG TGATCCGCTG ATCGACGCCA ATACCGGTCC CGGTGACAGC 720  
 25 GGCAATACCC GTGGTGAAGC GGGGCAACTG ATCGGCGAGC TTATCGACCG TGGCCTGCAA 780  
 TCGGTATTGG CCGGTGGTGG ACTGGGCACA CCCGTAAACA CCCCAGAC CCGTACGTCG 840  
 30 GCGAATGGCG GACAGTCCGC TCAGGATCTT GATCAGTTGC TGGGCGGCTT GCTGCTCAAG 900  
 GGCCTGGAGG CAACGCTCAA GGATGCCGGG CAAACAGGCA CCGACGTGCA GTCGAGCGCT 960  
 GCGCAAATCG CCACCTTGCT GGTCAGTACG CTGCTGCAAG GCACCCGCAA TCAGGCTGCA 1020  
 35 GCCTGA

1026

The hypersensitive response elicitor  
 polypeptide or protein derived from *Pseudomonas*  
 40 *solanacearum* has an amino acid sequence corresponding to  
 SEQ. ID. No. 7 as follows:

Met Ser Val Gly Asn Ile Gln Ser Pro Ser Asn Leu Pro Gly Leu Gln  
 1 5 10 15  
 45 Asn Leu Asn Leu Asn Thr Asn Thr Asn Ser Gln Gln Ser Gly Gln Ser  
 20 25 30  
 50 Val Gln Asp Leu Ile Lys Gln Val Glu Lys Asp Ile Leu Asn Ile Ile  
 35 40 45  
 Ala Ala Leu Val Gln Lys Ala Ala Gln Ser Ala Gly Gly Asn Thr Gly  
 50 55 60  
 55 Asn Thr Gly Asn Ala Pro Ala Lys Asp Gly Asn Ala Asn Ala Gly Ala  
 65 70 75 80  
 Asn Asp Pro Ser Lys Asn Asp Pro Ser Lys Ser Gln Ala Pro Gln Ser  
 85 90 95

- 24 -

Ala Asn Lys Thr Gly Asn Val Asp Asp Ala Asn Asn Gln Asp Pro Met  
 100 105 110  
 5 Gln Ala Leu Met Gln Leu Leu Glu Asp Leu Val Lys Leu Leu Lys Ala  
 115 120 125  
 Ala Leu His Met Gln Gln Pro Gly Gly Asn Asp Lys Gly Asn Gly Val  
 130 135 140  
 10 Gly Gly Ala Asn Gly Ala Lys Gly Ala Gly Gly Gln Gly Gly Leu Ala  
 145 150 155 160  
 Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly  
 165 170 175  
 15 Gly Ala Gly Ala Gly Gly Ala Gly Gly Val Gly Gly Ala Gly Gly  
 180 185 190  
 20 Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala  
 195 200 205  
 Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn  
 210 215 220  
 25 Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp  
 225 230 235 240  
 Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn  
 245 250 255  
 30 Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Gly Asn Gln  
 260 265 270  
 35 Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly  
 275 280 285  
 Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser  
 290 295 300  
 40 Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val  
 305 310 315 320  
 Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln  
 325 330 335  
 45 Gln Ser Thr Ser Thr Gln Pro Met  
 340

50 It is encoded by a DNA molecule having a nucleotide sequence corresponding SEQ. ID. No. 8 as follows:

	ATGTCAGTCG GAAACATCCA GAGCCCGTCG AACCTCCCGG GTCTGCAGAA CCTGAACCTC	60
55	AACACCAACA CCAACAGCCA GCAATCGGGC CAGTCCGTGC AAGACCTGAT CAAGCAGGTC	120
	GAGAAGGACA TCCTCAACAT CATCGCAGCC CTCGTGCAGA AGGCCGCACA GTCGGCGGGC	180
60	GGCAACACCG GTAACACCGG CAACGCGCCG GCGAAGGACG GCAATGCCAA CGCGGGCGCC	240

- 25 -

AACGACCCGA GCAAGAACGA CCCGAGCAAG AGCCAGGCTC CGCAGTCGGC CAACAAGACC 300  
 GGCAACGTCG ACGACGCCAA CAACCAGGAT CCGATGCAAG CGCTGATGCA GCTGCTGGAA 360  
 5 GACCTGGTGA AGCTGCTGAA GGC GGCCCTG CACATGCAGC AGCCCGGCGG CAATGACAAG 420  
 GGCAACGGCG TGGGCGGTGC CAACGGCGCC AAGGGTGCCG GCGGCCAGGG CGGCCTGGCC 480  
 10 GAAGCGCTGC AGGAGATCGA GCAGATCCTC GCCCAGCTCG GCGGCGGCGG TGCTGGCGCC 540  
 GCGGCGGCGG GTGGCGGTGT CGGCGGTGCT GGTGGCGCGG ATGGCGGCTC CGGTGCGGGT 600  
 GGCGCAGGCG GTGCGAACGG CGCCGACGGC GGCAATGGCG TGAACGGCAA CCAGGCGAAC 660  
 15 GGCCCGCAGA ACGCAGGCGA TGTCAACGGT GCCAACGGCG CGGATGACGG CAGCGAAGAC 720  
 CAGGGCGGCC TCACCGGCGT GCTGCAAAAG CTGATGAAGA TCCTGAACGC GCTGGTGCAG 780  
 20 ATGATGCAGC AAGGCGGCCT CGGCGGCGGC AACCAGGCGC AGGGCGGCTC GAAGGGTGCC 840  
 GGCAACGCCT CGCCGGCTTC CGGCGCGAAC CCGGGCGCGA ACCAGCCCGG TTCGGCGGAT 900  
 GATCAATCGT CCGGCCAGAA CAATCTGCAA TCCCAGATCA TGGATGTGGT GAAGGAGGTC 960  
 25 GTCCAGATCC TGCAGCAGAT GCTGGCGGCG CAGAACGGCG GCAGCCAGCA GTCCACCTCG 1020  
 ACGCAGCCGA TGTA 1035

30 Further information regarding the hypersensitive response  
 elicitor polypeptide or protein derived from *Pseudomonas*  
*solanacearum* is set forth in Arlat, M., F. Van Gijsegem,  
 J. C. Huet, J. C. Pemollet, and C. A. Boucher, "PopA1, a  
 35 Protein which Induces a Hypersensitive-like Response in  
 Specific Petunia Genotypes, is Secreted via the Hrp  
 Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-533  
 (1994), which is hereby incorporated by reference.

The hypersensitive response elicitor  
 40 polypeptide or protein from *Xanthomonas campestris* pv.  
*glycines* has an amino acid sequence corresponding to SEQ.  
 ID. No. 9 as follows:

45 Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Ala Ile Leu Ala  
 1 5 10 15  
 Ala Ile Ala Leu Pro Ala Tyr Gln Asp Tyr  
 20 25

50

This sequence is an amino terminal sequence having 26 residues only from the hypersensitive response elicitor polypeptide or protein of *Xanthomonas campestris* pv. *glycines*. It matches with fimbrial subunit proteins determined in other *Xanthomonas campestris* pathovars.

The hypersensitive response elicitor polypeptide or protein from *Xanthomonas campestris pelargonii* is heat stable, protease sensitive, and has a molecular weight of 20kDa. It includes an amino acid sequence corresponding to SEQ. ID. No. 10 as follows:

Ser Ser Gln Gln Ser Pro Ser Ala Gly Ser Glu Gln Gln Leu Asp Gln  
 1 5 10 15  
 15 Leu Leu Ala Met  
 20

Isolation of *Erwinia carotovora* hypersensitive response elicitor protein or polypeptide is described in Cai, et al., "The RsmA<sup>-</sup> Mutants of *Erwinia carotovora* subsp. *carotova* Strain Ecc71 Overexpress *hrpN*<sub>Ecc</sub> and Elicit a Hypersensitive Reaction-Like Response in Tobacco Leaves," MPMI, 9(7):565-73 (1996), which is hereby incorporated by reference. The hypersensitive response elicitor protein or polypeptide for *Erwinia stewartii* is disclosed in Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microbe Interact, July 14-19, 1996 and Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc., July 27-31, 1996, which are hereby incorporated by reference.

Hypersensitive response elicitor proteins or polypeptides from *Phytophthora parasitica*, *Phytophthora cryptogea*, *Phytophthora cinnamoni*, *Phytophthora capsici*, *Phytophthora megasperma*, and *Phytophthora citrophthora* are described in Kamoun, et al., "Extracellular Protein Elicitors from *Phytophthora*: Host-Specificity and

Induction of Resistance to Bacterial and Fungal  
Phytopathogens," Molec. Plant-Microbe Interact., 6(1):15-  
25 (1993), Ricci, et al., "Structure and Activity of  
Proteins from Pathogenic Fungi *Phytophthora* Eliciting  
5 Necrosis and Acquired Resistance in Tobacco," Eur. J.  
Biochem., 183:555-63 (1989), Ricci, et al., "Differential  
Production of Parasiticein, an Elicitor of Necrosis and  
Resistance in Tobacco by Isolates of *Phytophthora*  
*paraticica*," Plant Path., 41:298-307 (1992), Baillieul,  
10 et al., "A New Elicitor of the Hypersensitive Response in  
Tobacco: A Fungal Glycoprotein Elicits Cell Death,  
Expression of Defense Genes, Production of Salicylic  
Acid, and Induction of Systemic Acquired Resistance,"  
Plant J., 8(4):551-60 (1995), and Bonnet, et al.,  
15 "Acquired Resistance Triggered by Elicitins in Tobacco  
and Other Plants," Eur. J. Plant Path., 102:181-92  
(1996), which are hereby incorporated by reference.

The above elicitors are exemplary. Other  
elicitors can be identified by growing fungi or bacteria  
20 that elicit a hypersensitive response under which genes  
encoding an elicitor are expressed. Cell-free  
preparations from culture supernatants can be tested for  
elicitor activity (i.e. local necrosis) by using them to  
infiltrate appropriate plant tissues.

25 It is also possible to use fragments of the  
above hypersensitive response elicitor polypeptides or  
proteins as well as fragments of full length elicitors  
from other pathogens, in the method of the present  
invention.

30 Suitable fragments can be produced by several  
means. In the first, subclones of the gene encoding a  
known elicitor protein are produced by conventional  
molecular genetic manipulation by subcloning gene  
fragments. The subclones then are expressed *in vitro* or  
35 *in vivo* in bacterial cells to yield a smaller protein or

a peptide that can be tested for elicitor activity according to the procedure described below.

As an alternative, fragments of an elicitor protein can be produced by digestion of a full-length elicitor protein with proteolytic enzymes like chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave elicitor proteins at different sites based on the amino acid sequence of the elicitor protein. Some of the fragments that result from proteolysis may be active elicitors of resistance.

In another approach, based on knowledge of the primary structure of the protein, fragments of the elicitor protein gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for increased expression of a truncated peptide or protein.

An example of a suitable fragment is the popA1 fragment of the hypersensitive response elicitor polypeptide or protein from *Pseudomonas solanacearum*. See Arlat, M., F. Van Gijsegem, J.C. Huet, J.C. Pemollet, and C.A. Boucher, "PopA1, a Protein Which Induces a Hypersensitive-like Response in Specific *Petunia* Genotypes is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," *EMBO J.* 13:543-53 (1994), which is hereby incorporated by reference. As to *Erwinia amylovora*, a suitable fragment can be, for example, either or both the polypeptide extending between and including amino acids 1 and 98 of SEQ. ID. NO. 3 and the polypeptide extending between and including amino acids 137 and 204 of SEQ. ID. No. 3.

Variants may be made by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and

- 29 -

hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide.

The protein or polypeptide of the present invention is preferably produced in purified form (preferably at least about 60%, more preferably 80%, pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is produced but not secreted into the growth medium of recombinant *E. coli*. Alternatively, the protein or polypeptide of the present invention is secreted into the growth medium. In the case of unsecreted protein, to isolate the protein, the *E. coli* host cell carrying a recombinant plasmid is propagated, homogenized, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to heat treatment and the hypersensitive response elicitor protein is separated by centrifugation. The supernatant fraction containing the polypeptide or protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by ion exchange or HPLC.

Alternatively, the hypersensitive response elicitor protein can be prepared by chemical synthesis using conventional techniques.

The DNA molecule encoding the hypersensitive response elicitor polypeptide or protein can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA

- 30 -

molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the  
5 necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of  
10 recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.  
15 Recombinant genes may also be introduced into viruses, such as vaccina virus. Recombinant viruses can be generated by transection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited  
20 to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning  
25 Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes,"  
30 Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA  
35 sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook



- 31 -

et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

A variety of host-vector systems may be  
5 utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with  
10 bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems  
15 infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events  
20 control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby  
25 promotes mRNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further,  
30 procaryotic promoters are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient  
35 translation of mRNA in procaryotes requires a ribosome

- 32 -

binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promoters vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter, *trp* promoter, *recA* promoter, ribosomal RNA promoter, the  $P_R$  and  $P_L$  promoters of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lacUV5* (*tac*) promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the

- 33 -

addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required  
5 for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector,  
10 which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires a Shine-Dalgarno (SD) sequence about 7-9 bases 5' to the initiation codon (ATG)  
15 to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli*  
20 tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding the  
25 hypersensitive response elicitor polypeptide or protein has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system.  
30 Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The method of the present invention can be utilized to treat seeds for a wide variety of plants to  
35 impart pathogen resistance to the plants. Suitable seeds

are for plants which are dicots and monocots. More particularly, useful crop plants can include: rice, wheat, barley, rye, oats, cotton, sunflower, canola, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Examples of suitable ornamental plants are: rose, *Saintpaulia*, petunia, *Pelargonium*, poinsettia, chrysanthemum, carnation, and zinnia.

The method of imparting pathogen resistance to plants in accordance with the present invention is useful in imparting resistance to a wide variety of pathogens including viruses, bacteria, and fungi.

Resistance, *inter alia*, to the following viruses can be achieved by the method of the present invention: Tobacco mosaic virus, cucumber mosaic virus, potato x virus, potato y virus, and tomato mosaic virus.

Resistance, *inter alia*, to the following bacteria can also be imparted to plants in accordance with the present invention: *Pseudomonas solancearum*, *Pseudomonas syringae* pv. *tabaci*, and *Xanthomonas campestris* pv. *pelargonii*.

Plants can be made resistant, *inter alia*, to the following fungi by use of the method of the present invention: *Fusarium oxysporum* and *Phytophthora infestans*.

The embodiment of the present invention involving applying the hypersensitive response elicitor polypeptide or protein to all or part of the plant seeds being treated can be carried out through a variety of procedures. Suitable application methods include high or low pressure spraying, injection, coating, dusting, and

- 35 -

immersion. Other suitable application procedures can be envisioned by those skilled in the art. Once treated with the hypersensitive response elicitor of the present invention, the seeds can be planted and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants may be treated with one or more applications of the hypersensitive response elicitor protein or polypeptide to enhance hypersensitive response induced resistance in the plants. See U.S. Patent Application Serial No. 08/475,775, which is hereby incorporated by reference. Such propagated plants, which are resistant to disease, may, in turn, be useful in producing seeds or propagules (e.g. cuttings) that produce resistant plants.

The hypersensitive response elicitor polypeptide or protein can be applied to plant seeds in accordance with the present invention alone or in a mixture with other materials.

A composition suitable for treating plant seeds in accordance with the present invention contains a hypersensitive response elicitor polypeptide or protein in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition contains greater than 0.5 nM hypersensitive response elicitor polypeptide or protein.

Although not required, this composition may contain additional additives including fertilizer, insecticide, fungicide, nematocide, herbicide, and mixtures thereof. Suitable fertilizers include  $(\text{NH}_4)_2\text{NO}_3$ . An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the

process of the present invention. In addition, the hypersensitive response elicitor polypeptide or protein can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

In the alternative embodiment of the present invention involving the use of transgenic seeds, a hypersensitive response elicitor polypeptide or protein need not be applied topically to the seeds. Instead, transgenic plants transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein are produced according to procedures well known in the art, such as biolistics or *Agrobacterium* mediated transformation. Examples of suitable hypersensitive response elicitor polypeptides or proteins and the nucleic acid sequences for their encoding DNA are disclosed *supra*. As is conventional in the art, such transgenic plants would contain suitable vectors with various promoters including pathogen-induced promoters, and other components needed for transformation, transcription, and, possibly, translation. Such transgenic plants themselves could be grown under conditions effective to be imparted with pathogen resistance. In any event, once transgenic plants of this type are produced, transgenic seeds are recovered. These seeds can then be planted in the soil and cultivated using conventional procedures to produce plants. The plants are propagated from the planted transgenic seeds under conditions effective to impart pathogen resistance to the plants.

When transgenic plant seeds are used in accordance with the present invention, they additionally can be treated with the same materials (noted above) as are used to treat the seeds to which a hypersensitive response elicitor polypeptide or protein is applied.

- 37 -

These other materials, including hypersensitive response elicitors, can be applied to the transgenic plant seeds by high or low pressure spraying, injection, coating, dusting, and immersion. Similarly, transgenic plants additionally may be treated with one or more applications of the hypersensitive response elicitor to enhance hypersensitive response induced resistance in the plants. Such plants may also be treated with conventional plant treatment agents (e.g., insecticides, fertilizers, etc.). The transgenic plants of the present invention are useful in producing seeds or propagules (e.g. cuttings) from which disease resistant plants grow.

#### EXAMPLES

##### Example 1 - Effect of Treating Seeds with Hypersensitive Response Elicitor Protein

Marglobe tomato seeds were submerged in hypersensitive response elicitor protein (ca. 26  $\mu\text{gm/ml}$ ) from *Erwinia amylovora* solution or buffer in beakers on day 0 for 24 hours at 28°C in a growth chamber. After soaking seeds in hypersensitive response elicitor protein from *Erwinia amylovora* or buffer, they were sown in germination pots with artificial soil on day 1. Seedlings were transplanted to individual pots at the two-true-leaf stage on day 12. After transplanting, some plants that arose from treated seed also were sprayed with hypersensitive response elicitor protein (ca. 13  $\mu\text{gm/ml}$ ) from *Erwinia amylovora* (Treatments 3 and 4).

Tomato treated as noted in the preceding paragraph were inoculated with *Burkholderia* (*Pseudomonas*) *solanacearum* K60 strain (See Kelman, "The Relationship of Pathogenicity in *Pseudomonas solanacearum* to Colony Appearance on a Tetrazolium Medium," Phytopathology 44:693-95 (1954)) on day 23 by making vertical cuts

through the roots and potting medium of tomato plants (on a tangent 2 cm from the stem and 2 times/pot) and putting 10 ml ( $5 \times 10^8$  cfu/ml) suspension into the soil.

The above procedure involved use of 10 seeds treated with hypersensitive response elicitor protein from *Erwinia amylovora* per treatment.

#### Treatments:

- 
- |    |   |
|----|---|
| 10 | 1. Seeds soaked in hypersensitive response elicitor protein from <i>Erwinia amylovora</i> (ca. 26 $\mu$ mg/ml).   |
|    | 2. Seeds soaked in buffer (5mM KPO <sub>4</sub> , pH 6.8).  |
| 15 | 3. Seeds soaked in hypersensitive response elicitor protein from <i>Erwinia amylovora</i> (ca. 26 $\mu$ mg/ml) and seedlings sprayed with hypersensitive response elicitor protein from <i>Erwinia amylovora</i> (ca. 13 $\mu$ gm/ml) at transplanting. |
| 20 | 4. Seeds soaked in buffer and seedlings sprayed with hypersensitive response elicitor protein from <i>Erwinia amylovora</i> (ca. 13 $\mu$ gm/ml) at transplanting.  |

25 The results of these treatments are set forth in Tables 1-4:



- 39 -

Table 1 - Infection Data - 28 Days After Seed  
Treatment and 5 Days After Inoculation

Treatm.	Plants	Number of Plants of Given Disease Rating*					
		0	1	2	3	4	5
1	10	10	0	0	0	0	0
2	10	9	1	0	0	0	0
3	10	9	1	0	0	0	0
4	10	10	0	0	0	0	0

\* Disease Scale:

Grade 0: No symptoms

Grade 1: One leaf partially wilted.

Grade 2: 2-3 leaves wilted.

Grade 3: All except the top 2-3 leaves wilted.

Grade 4: All leaves wilted.

Grade 5: Plant Dead

Table 2 - Infection Data - 31 Days After Seed  
Treatment and 8 Days After Inoculation

Treatm.	Plants	Number of Plants of Given Disease Rating*					
		0	1	2	3	4	5
1	10	6	4	0	0	0	0
2	10	4	3	2	1	0	0
3	10	8	2	0	0	0	0
4	10	7	2	1	0	0	0

- 40 -

Table 3 - Infection Data - 35 Days After Seed Treatment and 12 Days After Inoculation

		Number of Plants of Given Disease Rating*						
5	Treatm.	Plants	0	1	2	3	4	5
	1	10	5	3	0	1	1	0
	2	10	1	3	3	2	1	0
	3	10	4	3	3	0	0	0
	4	10	3	3	3	1	0	0

Table 4 - Disease Indices of Seed Treatment With Hypersensitive Response Elicitor Protein

15	Treatment		Inoculation	Disease Index (%) *		
	Day 0	Day 14	Day 23	Day 28	Day 31	Day 35
20	1. Hypersensitive response elicitor protein seed soak		Inoculate	0	8	20
	2. Buffer seed soak		Inoculate	2	20	38
25	3. Hypersensitive response elicitor protein seed soak	Spray Hypersensitive response elicitor protein	Inoculate	2	4	18
30	4. Buffer seed soak	Spray Hypersensitive response elicitor protein	Inoculate	0	8	24

35 \* The Disease Index was determined using the procedure set forth in N.N. Winstead, et al., "Inoculation Techniques for Evaluating Resistance to *Pseudomonas Solanacearum*," Phytopathology 42:628-34 (1952), particularly at page 629.

- 41 -

The above data shows that the hypersensitive response elicitor protein was more effective than buffer as a seed treatment in reducing disease index and was as effective as spraying leaves of young plants with hypersensitive response elicitor protein.

**Example 2 - Effect of Treating Tomato Seeds With Hypersensitive Response Elicitor Protein From pCPP2139 Versus pCPP50 Vector On Southern Bacteria Wilt Of Tomato**

Marglobe tomato seeds were submerged in hypersensitive response elicitor protein from pCPP2139 or in pCPP50 vector solution (1:50, 1:100 and 1:200) in beakers on day 0 for 24 hours at 28°C in a growth chamber. After soaking seeds in hypersensitive response elicitor protein or vector, they were sown in germination pots with artificial soil on day 0. Ten uniform appearing plants were chosen randomly from each of the following treatments:

Treatment Content	Strain	Dilution	Harpin
25	1. DH5 $\alpha$ (pCPP2139)	1:50	8 $\mu$ g/ml
	2. DH5 $\alpha$ (pCCP50)	1:50	0
	3. DH5 $\alpha$ (pCPP2139)	1:100	4 $\mu$ g/ml
	4. DH5 $\alpha$ (pCPP50)	1:100	0
	5. DH5 $\alpha$ (pCPP2139)	1:200	2 $\mu$ g/ml
30	6. DH5 $\alpha$ (pCPP50)	1:200	0

The resulting seedlings were inoculated with *Pseudomonas solanacearum* K60 by dipping the roots of tomato seedling plants for about 30 seconds in a 40 ml ( $1 \times 10^8$  cfu/ml) suspension. The seedlings were then transplanted into the pots with artificial soil on day 12.

The results of these treatments are set forth in Tables 5-8.

Table 5 - 16 Days After Seed Treatment and  
3 Days After Inoculation

5

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	7	3	0	0	0	0
2	10	5	5	0	0	0	0
3	10	6	4	0	0	0	0
4	10	6	4	0	0	0	0
5	10	7	4	0	0	0	0
6	10	4	6	0	0	0	0

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15 Table 6 - 19 Days After Seed Treatment and  
6 Days After Inoculation

20

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	6	0	0	0	0	0
2	10	2	0	2	2	1	3
3	10	2	0	2	0	2	4
3	10	3	1	2	0	2	2
5	10	2	1	0	2	2	3
6	10	1	0	1	1	3	4

25

Table 7 - 21 Days After Seed Treatment and  
8 Days After Inoculation

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	6	0	0	0	4	3
2	10	2	0	0	1	4	4
3	10	2	0	0	2	2	3
4	10	3	0	0	2	2	3
5	10	2	0	0	0	4	4
6	10	1	0	1	2	1	5

Table 8 - Disease Indices of Seed Treatment  
With Hypersensitive Response Elicitor and Vector

Treatment		Disease Index (%)		
Day 0	Day 12	Day 15	Day 18	Day 20
Hypersensitive response elicitor protein seed dip (1:50)	inoculate	6.0	32.0	38.0
Vector seed dip (1:50)	inoculate	10.0	58.0	70.0
Hypersensitive response elicitor protein seed dip (1:100)	inoculate	8.0	64.0	68.0
Vector seed dip (1:100)	inoculate	8.0	46.0	58.0
Hypersensitive response elicitor protein seed dip (1:200)	inoculate	6.0	60.00	72.0
Vector seed dip (1:200)	inoculate	12.0	74.0	74.0

The above data shows that the hypersensitive response elicitor protein is much more effective than the vector solution in preventing Tomato Southern Bacteria Wilt.

- 44 -

**Example 3 - Effect of Treating Tomato Seeds With Hypersensitive Response Elicitor Protein From pCPP2139 Versus pCPP50 Vector On Tomato Southern Bacteria Wilt**

5 Marglobe tomato seeds were submerged in hypersensitive response elicitor protein from pCPP2139 or in pCPP50 vector solution (1:50, 1:100 and 1:200) in beakers on day 0 for 24 hours at 28°C in a growth  
10 chamber. After soaking seeds in the hypersensitive response elicitor protein or vector, the seeds were sown in germination pots with artificial soil on day 1. Ten uniform appearing plants were chosen randomly from each of the following treatments:

15	Treatment	Strain	Dilution	Hypersensitive Response Elicitor Content
20	1.	DH5α(pCPP2139)	1:50	8 µg/ml
	2.	DH5α(pCPP50)	1:50	0
	3.	DH5α(pCPP2139)	1:100	4 µg/ml
	4.	DH5α(pCPP50)	1:100	0
	5.	DH5α(pCPP2139)	1:200	2 µg/ml
25	6.	DH5α(pCPP50)	1:200	0

The resulting seedlings were inoculated with *Pseudomonas solanacearum* K60 by dipping the roots of tomato seedling plants  
30 for about 30 seconds in a 40 ml ( $1 \times 10^6$  cfu/ml) suspension. The seedlings were then transplanted into the pots with artificial soil on day 12.

The results of these treatments are set forth in Tables 9-12.

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- 45 -

Table 9 - 16 Days After Seed Treatment and  
3 Days After Inoculation

5			Number of Plants of Given Disease Rating*					
	Treatm.	Plants	0	1	2	3	4	5
	1	10	8	2	0	0	0	0
	2	10	7	3	0	0	0	0
	3	10	7	3	0	0	0	0
	4	10	7	3	0	0	0	0
	5	10	8	2	0	0	0	0
	6	10	7	3	0	0	0	0

Table 10 - 19 Days After Seed Treatment and  
6 Days After Inoculation

		Number of Plants of Given Disease Rating*						
	Treatm.	Plants	0	1	2	3	4	5
20	1	10	5	0	0	1	2	2
	2	10	1	0	1	2	3	3
	3	10	4	1	0	0	2	3
	4	10	2	0	2	1	2	3
25	5	10	1	0	1	1	4	3
	6	10	1	0	0	2	4	3

**Table 11 - 21 Days After Hypersensitive Response  
Elicitor Protein Seed Treatment and  
8 Days After Inoculation**

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	5	0	0	0	2	3
2	10	2	0	2	0	2	4
3	10	5	0	0	0	2	3
4	10	2	0	2	0	2	4
5	10	1	0	1	0	2	6
6	10	1	0	0	0	2	7

**Table 12 - Disease Indices of Seed Treatment  
With Hypersensitive Response Elicitor Protein and Vector**

Day 1	Day 13	Day 16	Day 19	Day 21
Hypersensitive response elicitor protein seed dip (1:50)	inoculate	4.0	42.0	46.0
Vector seed dip (1:50)	inoculate	6.0	70.0	64.0
Hypersensitive response elicitor protein seed dip (1:100)	inoculate	6.0	48.0	46.0
Vector seed dip (1:100)	inoculate	6.0	60.0	64.0
Hypersensitive response elicitor protein seed dip (1:200)	inoculate	4.0	72.0	80.0
Vector seed dip (1:200)	inoculate	6.0	74.0	86.0

The above data shows that the hypersensitive response elicitor protein is much more effective in preventing Tomato Southern Bacteria Wilt.



**Example 4 - Effect of Treating Tomato Seeds With Hypersensitive Response Elicitor Protein From pCPP2139 Versus pCPP50 Vector On Southern Bacteria Wilt Of Tomato**

Marglobe tomato seeds were submerged in hypersensitive response elicitor protein from pCPP2139 or in pCPP50 vector solution (1:25, 1:50 and 1:100) in beakers on day 0 for 24 hours at 28°C in a growth chamber. After soaking seeds in hypersensitive response elicitor protein or vector, they were sown in germination pots with artificial soil on day 1. Ten uniform appearing plants were chosen randomly from each of the following treatments:

Treatment Content	Strain	Dilution	Harpin
1.	DH5 $\alpha$ (pCPP2139)	1:25	16 $\mu$ g/ml
2.	DH5 $\alpha$ (pCPP50)	1:25	0
3.	DH5 $\alpha$ (pCPP2139)	1:50	8 $\mu$ g/ml
4.	DH5 $\alpha$ (pCPP50)	1:50	0
5.	DH5 $\alpha$ (pCPP2139)	1:100	2 $\mu$ g/ml
6.	DH5 $\alpha$ (pCPP50)	1:100	0

The resulting seedlings were inoculated with *Pseudomonas solanacearum* K60 by dipping the roots of tomato seedling plants for about 30 seconds in a 40 ml ( $1 \times 10^8$  cfu/ml) suspension. The seedlings were then transplanted into the pots with artificial soil on day 14.

The results of these treatments are set forth in Tables 13-16.

Table 13 - 19 Days After Seed Treatment and  
4 Days After Inoculation

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	8	2	0	0	0	0
2	10	5	2	2	1	0	0
3	10	9	1	0	0	0	0
4	10	5	2	1	2	0	0
5	10	5	3	1	1	0	0
6	10	6	1	2	1	0	0

Table 14 - 21 Days After Seed Treatments and  
6 Days After Inoculation

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	6	3	0	0	1	0
2	10	3	2	1	0	0	0
3	10	6	3	1	0	0	0
4	10	3	2	1	2	2	0
5	10	5	1	2	2	0	0
6	10	3	1	3	2	1	0

- 49 -

Table 15 - 23 Days After Seed Treatment and  
8 Days After Inoculation

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	7	2	0	0	0	1
2	10	2	2	2	3	0	1
3	10	7	2	0	1	0	0
4	10	2	1	2	3	0	2
5	10	3	1	2	3	0	1
6	10	2	2	2	3	0	1

Table 16 - Disease Indices of Seed Treatment  
With Hypersensitive Elicitor Protein and Vector

Treatment		Disease Index (%)		
Day 1	Day 15	Day 19	Day 21	Day 23
Hypersensitive response elicitor protein seed dip (1:25)	inoculate	4.0	14.0	14.0
Vector seed dip (1:25)	inoculate	18.0	28.0	40.0
Hypersensitive response elicitor protein seed dip (1:50)	inoculate	2.0	10.0	10.0
Vector seed dip (1:50)	inoculate	20.0	36.0	48.0
Hypersensitive response elicitor protein seed dip (1:100)	inoculate	16.0	22.0	38.0
Vector seed dip (1:100)	inoculate	16.0	34.0	40.0

The above data shows that the hypersensitive response elicitor protein is much more effective than the vector solution in preventing Tomato Southern Bacteria

Wilt. A hypersensitive response protein concentration of 1:50 is particularly effective in disease control.

5 Example 5 - Effect of Treating Tomato Seeds With  
Hypersensitive Response Elicitor Protein  
From pCPP2139 Versus pCPP50 Vector On  
Southern Bacteria Wilt Of Tomato

10 Marglobe tomato seeds were submerged in  
hypersensitive response elicitor protein from pCPP2139 or  
pCPP50 vector solution (1:25, 1:50 and 1:100) in beakers  
on day 0 for 24 hours at 28°C in a growth chamber. After  
soaking seeds in hypersensitive response elicitor protein  
or vector, they were sown in germination pots with  
15 artificial soil on day 1. Ten uniform appearing plants  
were chosen randomly from each of the following  
treatments:

20	Treatment Content	Strain	Dilution	Harpin
	1.	DH5α(pCPP2139)	1:25	16 µg/ml
	2.	DH5α(pCPP50)	1:25	0
	3.	DH5α(pCPP2139)	1:50	8 µg/ml
25	4.	DH5α(pCPP50)	1:50	0
	5.	DH5α(pCPP2139)	1:100	4 µg/ml
	6.	DH5α(pCPP50)	1:100	0

30 The resulting seedlings were inoculated with *Pseudomonas solanacearum* K60 by dipping the roots of tomato seedling plants for about 30 seconds in a 40 ml ( $1 \times 10^6$  cfu/ml) suspension. The seedlings were then transplanted into the pots with artificial soil on day 14.

35 The results of these treatments are set forth in Tables 17-20.

- 51 -

Table 17 - 19 Days After Seed Treatment and  
4 Days After Inoculation

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	8	2	0	0	0	0
2	10	6	3	1	0	0	0
3	10	9	1	0	0	0	0
4	10	6	4	0	0	0	0
5	10	6	2	1	1	0	0
6	10	6	4	0	0	0	0

Table 18 - 21 Days After Seed Treatment and  
6 Days After Inoculation

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	7	2	1	1	0	0
2	10	3	3	2	2	0	0
3	10	8	2	0	0	0	0
4	10	3	2	2	2	0	0
5	10	6	1	1	2	0	0
6	10	3	2	3	1	1	0

Table 19 - 23 Days After Seed Treatment and  
8 Days After Inoculation

		Number of Plants of Given Disease Rating*					
Treatm.	Plants	0	1	2	3	4	5
1	10	7	0	2	1	0	0
2	10	3	1	2	3	0	1
3	10	8	1	0	1	0	0
4	10	3	3	1	2	0	1
5	10	3	3	0	2	1	1
6	10	3	2	0	3	0	2

Table 20 - Disease Indices of Seed Treatment  
With Hypersensitive Response Elicitor Protein and Vector

Treatment		Disease Index (%)		
Day 0	Day 15	Day 19	Day 21	Day 23
Hypersensitive response elicitor protein seed dip (1:25)	inoculate	4.0	12.0	14.0
Vector seed dip (1:25)	inoculate	10.0	26.0	38.0
Hypersensitive response elicitor protein seed dip (1:50)	inoculate	2.0	4.0	8.0
Vector seed dip (1:50)	inoculate	8.0	26.0	32.0
Hypersensitive response elicitor protein seed dip (1:100)	inoculate	14.0	18.0	36.0
Vector seed dip (1:100)	inoculate	8.0	30.0	42.0

The above data shows that the hypersensitive response elicitor protein is much more effective than the

- 53 -

vector solution in preventing Tomato Southern Bacteria Wilt. A hypersensitive response elicitor protein concentration of 1:50 is more effective in disease control.

5

**Example 6 - Treating Rice Seeds with Hypersensitive Response Elicitor Protein to Reduce Rice Stem Rot**

10 Rice seeds (variety, M-202) were submerged in two gallons of hypersensitive response elicitor protein solution at a concentration of 20  $\mu$ g for 24 hours at room temperature. Rice seeds submerged in the same solution without hypersensitive response elicitor protein were  
15 used as a control. After soaking, the seeds were sown in a rice field by air plane spray. There were four replicates for both hypersensitive response elicitor protein and control treatment. The lot size of each replicate is 150 Ft<sup>2</sup>. The design of each plot was  
20 completely randomized, and each plot had substantial level contamination of *Sclerotium oryzae*. Three months after sowing, stem rot was evaluated according to the following rating scale: Scale 1 = no disease, 2 =  
25 disease present on the exterior of the leaf sheath, 3 = disease penetrates leaf sheath completely but is not present on culm, 4 = disease is present on culm exterior but does not penetrate to interior of culm, and 5 =  
30 disease penetrates to interior of culm. 40 plants from each replicate were sampled and assessed for the disease incidence and severity. From Table 21, it is apparent that treating seeds with hypersensitive response elicitor reduced both disease incidence and severity. More particularly, regarding incidence, 67% of the plants were infected by stem rot for the control treatment, however,  
35 only 40% plants were infected for the hypersensitive response elicitor protein treatment. As to severity, the disease index\* for the hypersensitive response elicitor

protein treatment was 34% and 60% for the control. Accordingly, treating rice seed with hypersensitive response elicitor protein resulted in a significant reduction of stem rot disease. The hypersensitive response elicitor protein-induced resistance in rice can last a season long. In addition to disease resistance, it was also observed that hypersensitive response elicitor protein-treated rice had little or no damage by army worm (*Spodoptera praefica*). In addition, the treated plants were larger and had deeper green color than the control plants.

Table 21 - Incidence and Severity of Stem Rot (*Schlerotium oryzae*) on Rice, M-202

Treatment	% plants given disease rating					Disease index (%) (severity)
	1	2	3	4	5	
Harpin 20 µg/ml	60	5	8	18	10	34
Control	33	5	18	28	18	60

\*Disease Index (%) for the harpin treatment

$$= \frac{1 \times 60 + 2 \times 5 + 3 \times 8 + 4 \times 18 + 5 \times 10}{5 \times 100} \times 100/100$$

\*Disease Index (%) for the control treatment

$$= \frac{1 \times 33 + 2 \times 5 + 3 \times 18 + 4 \times 28 + 5 \times 18}{5 \times 100 \times 100/100} \times 100/100$$



- 55 -

Example 7 - Effect of Treating Onion Seed with  
Hypersensitive Response Elicitor Protein  
on the Development of Onion Smut Disease  
(*Urocystis cepulae*) and On Seedling  
Emergence

5

Onion seed, variety Pennant, (Seed Lot# 64387),  
obtained from the Crookham Co., Caldwell, ID 83606,  
10 treated with hypersensitive response elicitor protein or  
a control was planted in a natural organic or "muck"  
soil. Some of the seedlings that grew from the sown seed  
were healthy, some had lesions characteristic of the  
Onion Smut disease, and some of the sown seed did not  
15 produce seedlings that emerged from the soil. Thus, the  
effect of treating onion seed with various concentrations  
of hypersensitive response elicitor protein was  
determined.

Naturally infested muck soil was obtained from  
20 a field in Oswego County, NY, where onions had been grown  
for several years and where the Onion Smut disease  
commonly had been problematic. Buckets of muck (5-gallon  
plastic) were stored at 4°C until used. The soil was  
mixed, sieved, and put in plastic flats 10 inches wide,  
25 20 inches long, and 2 inches deep for use in the tests  
described. Based on preliminary experiments, the soil  
contained many propagules of the Onion Smut fungus,  
*Urocystis cepulae*, such that when onion seed was sown in  
the soil, smut lesions developed on many of the seedlings  
30 that emerged from the soil. In addition, the soil  
harbored other microorganisms, including those that cause  
the "damping-off" disease. Among the several fungi that  
cause damping off are *Pythium*, *Fusarium*, and *Rhizoctonia*  
species.

35 The hypersensitive response elicitor protein  
encoded by the *hrpN* gene of *Erwinia amylovora* was used to  
treat seeds. It was produced by fermentation of the  
cloned gene in a high-expression vector in *E. coli*.  
Analysis of the cell-free elicitor preparation by high-

pressure liquid chromatography indicated its hypersensitive response elicitor protein content and on that basis appropriate dilutions were prepared in water. Seeds were soaked in a beaker containing hypersensitive response elicitor protein concentrations of 0, 5, 25, and 50  $\mu\text{gm/ml}$  of hypersensitive response elicitor protein for 24 hours. They were removed, dried briefly on paper towels, and sown in the muck soil. Treated seed was arranged by row, 15 seeds in each row for each treatment; each flat contained two replicates, and there were six replicates. Thus, a total of 90 seeds were treated with each concentration of hypersensitive response elicitor protein. The flats containing the seeds were held in a controlled environment chamber operating at 60°F (15.6°C), with a 14-hour day /10-hour night. Observations were made on seedling emergence symptoms (smut lesions). The data were recorded 23 days after sowing.

The effect of soaking onion seed in different concentrations of hypersensitive response elicitor protein on emergence of onion seedlings and on the incidence of onion smut is shown in Table 22. Only slight differences in emergence were noted, suggesting that there is no significant effect of treating with hypersensitive response elicitor protein at the concentrations used. Among the seedlings that emerged, substantially more of the seeds that received no hypersensitive response elicitor protein exhibited symptoms of Onion Smut than seedlings that grew from seed that had been treated with hypersensitive response elicitor protein. Treating seed with 25  $\mu\text{gm/ml}$  of hypersensitive response elicitor protein was the most effective concentration tested in reducing Onion Smut. Thus, this example demonstrates that treating onion seed with hypersensitive response elicitor protein reduces the Onion Smut disease.

Table 22 - Effect of Treating Onion Seed With Hypersensitive Response Elicitor Protein (i.e. Harpin) on the Development of Onion Smut Disease (*Urocystis cepulae*).

Treatment harpin ( $\mu\text{g/ml}$ )	Mean Seedlings Emerged (of 15)	Mean Percent Emerged	Emerged	
			Percent Healthy	Percent with Smut
0	5.00	33.3	20.0	80.0
5	3.67	24.4	40.9	59.1
25	4.33 <sup>1</sup>	28.8	50.0	46.2
50	4.17	27.7	44.0	56.0

<sup>1</sup> One seedling emerged then died.

**Example 8 -** Effect of Treating Tomato Seed with Hypersensitive Response Elicitor Protein on the Development of Bacterial Speck of Tomato (*Pseudomonas syringae* pv. *tomato*)

Tomato seed, variety New Yorker (Seed lot# 2273-2B), obtained from Harris Seeds, Rochester, NY, were treated with four concentrations of hypersensitive response elicitor protein (including a no-elicitor protein, water-treated control) and planted in peatlite soil mix. After 12 days and when the seedlings were in the second true-leaf stage, they were inoculated with the Bacterial Speck pathogen. Ten days later, the treated and inoculated plants were evaluated for extent of infection. Thus, the effect of treating tomato seed with various concentrations of hypersensitive response elicitor protein on resistance to *Pseudomonas syringae* pv. *tomato* was determined.

The hypersensitive response elicitor protein encoded by the *hrpN* gene of *Erwinia amylovora* was used to treat seeds. It was produced by fermentation of the cloned gene in a high-expression vector in *E. coli*.

Analysis of the cell-free elicitor preparation by high-pressure liquid chromatography indicated its hypersensitive response elicitor protein content and, on that basis, appropriate dilutions were prepared in water.

5 Seeds were soaked in a beaker containing hypersensitive response elicitor protein concentrations of 0, 5, 10, and 20  $\mu\text{gm/ml}$  of hypersensitive response elicitor protein for 24 hours. They were removed, dried briefly on paper towels, and sown. The soil was a mixture of peat and

10 Perlite™ in plastic flats 10 inches wide, 20 inches long, and 2 inches deep. Treated seed was arranged by row, 6 seeds in each row for each treatment; each flat contained two replicates, and there were four replicates and thus a total of 24 seeds that were treated with each  
15 concentration of hypersensitive response elicitor protein. The flats containing the seeds were held in a controlled environment chamber operating at 75°F (25°C), with a 14-hour day/10-hour night.

When twelve-days old, the tomato seedlings were  
20 inoculated with  $10^8$  colony forming units/ml of the pathogen, applied as a foliar spray. The flats containing the seedlings were covered with a plastic dome for 48 hours after inoculation to maintain high humidity. Observations were made on symptom severity using a  
25 rating scale of 0-5. The rating was based on the number of lesions that developed on the leaflets and the cotyledons and on the relative damage caused to the plant parts by necrosis that accompanied the lesions. The cotyledons and (true) leaflets were separately rated for  
30 disease severity 11 days after inoculation

The effect of soaking tomato seed in different concentrations of hypersensitive response elicitor protein (i.e. harpin) on the development of Bacterial Speck on leaflets and cotyledons of tomato is shown in  
35 Table 23. The seedlings that grew from seed treated with the highest amount of hypersensitive response elicitor

protein tested (20  $\mu\text{gm/ml}$ ) had fewer diseased leaflets and cotyledons than the treatments. The water-treated control seedlings did not differ substantially from the plants treated with the two lower concentrations of hypersensitive response elicitor protein. Considering the disease ratings, the results were similar. Only plants treated with the highest concentration of hypersensitive response elicitor protein had disease ratings that were less than those of the other treatments. This example demonstrates that treatment of tomato seed with hypersensitive response elicitor protein reduces the incidence and severity of Bacterial Speck of tomato.

**Table 23 - Effect of Treating Tomato Seed With Hypersensitive Response Elicitor Protein (i.e. Harpin) on the Subsequent Development of Bacterial Speck Disease (*Pseudomonas syringae* pv. *tomato*) on Tomato Cotyledons and Tomato Leaflets**

Treatment Harpin ( $\mu\text{g/ml}$ )	Cotyledons			Leaflets		
	Mean Diseased	Percent Diseased	Disease Rating	Mean Diseased	Percent Diseased	Disease Rating
0	6.0/9.0	66.6	0.8	25.8/68.8	37.5	0.5
5	5.3/7.3	72.4	0.8	22.5/68.0	37.5	0.5
10	5.8/8.0	72.3	0.8	25.5/66.0	38.6	0.5
20	5.3/8.5	61.8	0.6	23.8/73.5	32.3	0.4

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

- 60 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Cornell Research Foundation, Inc.
- (ii) TITLE OF INVENTION: HYPERSENSITIVE RESPONSE INDUCED  
RESISTANCE IN PLANTS BY SEED  
TREATMENT
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Nixon, Hargrave, Devans & Doyle LLP
  - (B) STREET: P.O. Box 1051, Clinton Square
  - (C) CITY: Rochester
  - (D) STATE: New York
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 14603
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 60/033,230
  - (B) FILING DATE: 05-DEC-1996
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Goldman, Michael L.
  - (B) REGISTRATION NUMBER: 30,727
  - (C) REFERENCE/DOCKET NUMBER: 19603/1202
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (716) 263-1304
  - (B) TELEFAX: (716) 263-1600

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 338 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear

- 61 -

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 20 25 30  
 Leu Gly Ser Ser Val Asp Lys Leu Ser Ser Thr Ile Asp Lys Leu Thr  
 35 40 45  
 Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu  
 50 55 60  
 Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser  
 65 70 75 80  
 Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys  
 85 90 95  
 Ser Gly Gly Asp Ala Leu Ser Lys Met Phe Asp Lys Ala Leu Asp Asp  
 100 105 110  
 Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln  
 115 120 125  
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 145 150 155 160  
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 Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala  
 195 200 205  
 Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val  
 210 215 220  
 Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp  
 225 230 235 240  
 Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp  
 245 250 255  
 Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys  
 260 265 270  
 Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln  
 275 280 285  
 Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr  
 290 295 300

- 62 -

Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala  
 305 310 315 320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala  
 325 330 335

Asn Ala

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2141 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CAGCAATATC	CCGGCATGTT	GCGCACGCTG	CTCGCTCGTC	GTTATCAGCA	GGCGGCAGAG	240
TGCGATGGCT	GCCATCTGTG	CCTGAACGGC	AGCGATGTAT	TGATCCTCTG	GTGGCCGCTG	300
CCGTCGGATC	CCGGCAGTTA	TCCGCAGGTG	ATCGAACGTT	TGTTTGAACCT	GGCGGGAATG	360
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CGATCATTAA	GATAAAGGCG	GCTTTTTTTTA	TTGCAAAACG	GTAACGGTGA	GGAACCGTTT	480
CACCGTCGGC	GTCACTCAGT	AACAAGTATC	CATCATGATG	CCTACATCGG	GATCGGCGTG	540
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AAATTACGATC	AAAGCGCACA	TCGGCGGTGA	TTTGGGCGTC	TCCGGTCTGG	GGCTGGGTGC	660
TCAGGGACTG	AAAGGACTGA	ATTCCGCGGC	TTCATCGCTG	GGTTCCAGCG	TGGATAAACT	720
GAGCAGCACC	ATCGATAAGT	TGACCTCCGC	GCTGACTTCG	ATGATGTTTG	GCGGCGCGCT	780
GGCGCAGGGG	CTGGGCGCCA	GCTCGAAGGG	GCTGGGGATG	AGCAATCAAC	TGGGCCAGTC	840
TTTCGGCAAT	GGCGCGCAGG	GTGCGAGCAA	CCTGCTATCC	GTACCGAAAT	CCGGCGGCGA	900
TGCGTTGTCA	AAAATGTTTG	ATAAAGCGCT	GGACGATCTG	CTGGGTCATG	ACACCGTGAC	960
CAAGCTGACT	AACCAGAGCA	ACCAACTGGC	TAATTCAATG	CTGAACGCCA	GCCAGATGAC	1020
CCAGGGTAAT	ATGAATGCGT	TCGGCAGCGG	TGTGAACAAC	GCACTGTCTG	CCATTCTCGG	1080
CAACGGTCTC	GGCCAGTCGA	TGAGTGGCTT	CTCTCAGCCT	TCTCTGGGGG	CAGGCGGCTT	1140
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- 63 -

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CAGATAGATT GCGGTTTCGT AATCAACATG GTAATGCGGT TCCGCCTGTG CGCCGGCCGG 1980
GATCACCACA ATATTCATAG AAAGCTGTCT TGCACCTACC GTATCGCGGG AGATACCGAC 2040
AAAATAGGGC AGTTTTTGCG TGGTATCCGT GGGGTGTTCC GGCCTGACAA TCTTGAGTTG 2100
GTCGTCATC ATCTTCTCC ATCTGGGCGA CCTGATCGGT T 2141

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## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 403 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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20           25           30
Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn
35           40           45
Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
50           55           60
Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
65           70           75           80

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- 64 -

Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu  
 85 90 95  
 Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr  
 100 105 110  
 Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro  
 115 120 125  
 Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser  
 130 135 140  
 Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln  
 145 150 155 160  
 Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly  
 165 170 175  
 Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu  
 180 185 190  
 Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly  
 195 200 205  
 Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly  
 210 215 220  
 Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu  
 225 230 235 240  
 Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln  
 245 250 255  
 Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln  
 260 265 270  
 Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe  
 275 280 285  
 Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met  
 290 295 300  
 Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro  
 305 310 315 320  
 Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser  
 325 330 335  
 Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn  
 340 345 350  
 Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn  
 355 360 365  
 Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp  
 370 375 380  
 Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu  
 385 390 395 400  
 Gly Ala Ala

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1288 base pairs

(B) TYPE: nucleic acid base

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAGCTTCGGC ATGGCACGTT TGACCGTTGG GTCGGCAGGG TACGTTTGAA TTATTCATAA	60
GAGGAATACG TTATGAGTCT GAATACAAGT GGGCTGGGAG CGTCAACGAT GCAAATTTCT	120
ATCGGCGGTG CGGGCGGAAA TAACGGGTTG CTGGGTACCA GTCGCCAGAA TGCTGGGTTG	180
GGTGGCAATT CTGCACTGGG GCTGGGCGGC GGTAATCAAA ATGATACCGT CAATCAGCTG	240
GCTGGCTTAC TCACCGGCAT GATGATGATG ATGAGCATGA TGGGCGGTGG TGGGCTGATG	300
GGCGGTGGCT TAGGCGGTGG CTTAGGTAAT GGCTTGGGTG GCTCAGGTGG CCTGGGCGAA	360
GGACTGTCTGA ACGCGCTGAA CGATATGTTA GGCGGTTTCGC TGAACACGCT GGGCTCGAAA	420
GGCGGCAACA ATACCACTTC AACAACAAAT TCCCCGCTGG ACCAGGCGCT GGGTATTAAC	480
TCAACGTCCC AAAACGACGA TTCCACCTCC GGCACAGATT CCACCTCAGA CTCCAGCGAC	540
CCGATGCAGC AGCTGCTGAA GATGTTTCAGC GAGATAATGC AAAGCCTGTT TGGTGATGGG	600
CAAGATGGCA CCCAGGGCAG TTCCTCTGGG GGCAAGCAGC CGACCGAAGG CGAGCAGAAC	660
GCCTATAAAA AAGGAGTCAC TGATGCGCTG TCGGGCCTGA TGGGTAATGG TCTGAGCCAG	720
CTCCTTGGCA ACGGGGGACT GGGAGGTGGT CAGGGCGGTA ATGCTGGCAC GGGTCTTGAC	780
GGTTCGTCGC TGGGCGGCAA AGGGCTGCAA AACCTGAGCG GGCCGGTGGA CTACCAGCAG	840
TTAGGTAACG CCGTGGGTAC CGGTATCGGT ATGAAAGCGG GCATTTCAGGC GCTGAATGAT	900
ATCGGTACGC ACAGGCACAG TTCAACCCGT TCTTTCGTCA ATAAAGGCGA TCGGGCGATG	960
GCGAAGGAAA TCGGTCAGTT CATGGACCAG TATCCTGAGG TGTTTGGCAA GCCGCAGTAC	1020
CAGAAAGGCC CGGGTCAGGA GGTGAAAACC GATGACAAAT CATGGGCAA AGCACTGAGC	1080
AAGCCAGATG ACGACGGAAT GACACCAGCC AGTATGGAGC AGTTCAACAA AGCCAAGGGC	1140
ATGATCAAAA GGCCCATGGC GGGTGATACC GGCAACGGCA ACCTGCAGGC ACGCGGTGCC	1200
GGTGGTTCTT CGCTGGGTAT TGATGCCATG ATGGCCGGTG ATGCCATTAA CAATATGGCA	1260
CTTGGCAAGC TGGGCGCGGC TTAAGCTT	1288

- 66 -

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 341 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

---

Met	Gln	Ser	Leu	Ser	Leu	Asn	Ser	Ser	Ser	Leu	Gln	Thr	Pro	Ala	Met	1	5	10	15
Ala	Leu	Val	Leu	Val	Arg	Pro	Glu	Ala	Glu	Thr	Thr	Gly	Ser	Thr	Ser	20	25	30	
Ser	Lys	Ala	Leu	Gln	Glu	Val	Val	Val	Lys	Leu	Ala	Glu	Glu	Leu	Met	35	40	45	
Arg	Asn	Gly	Gln	Leu	Asp	Asp	Ser	Ser	Pro	Leu	Gly	Lys	Leu	Leu	Ala	50	55	60	
Lys	Ser	Met	Ala	Ala	Asp	Gly	Lys	Ala	Gly	Gly	Gly	Ile	Glu	Asp	Val	65	70	75	80
Ile	Ala	Ala	Leu	Asp	Lys	Leu	Ile	His	Glu	Lys	Leu	Gly	Asp	Asn	Phe	85	90	95	
Gly	Ala	Ser	Ala	Asp	Ser	Ala	Ser	Gly	Thr	Gly	Gln	Gln	Asp	Leu	Met	100	105	110	
Thr	Gln	Val	Leu	Asn	Gly	Leu	Ala	Lys	Ser	Met	Leu	Asp	Asp	Leu	Leu	115	120	125	
Thr	Lys	Gln	Asp	Gly	Gly	Thr	Ser	Phe	Ser	Glu	Asp	Asp	Met	Pro	Met	130	135	140	
Leu	Asn	Lys	Ile	Ala	Gln	Phe	Met	Asp	Asp	Asn	Pro	Ala	Gln	Phe	Pro	145	150	155	160
Lys	Pro	Asp	Ser	Gly	Ser	Trp	Val	Asn	Glu	Leu	Lys	Glu	Asp	Asn	Phe	165	170	175	
Leu	Asp	Gly	Asp	Glu	Thr	Ala	Ala	Phe	Arg	Ser	Ala	Leu	Asp	Ile	Ile	180	185	190	
Gly	Gln	Gln	Leu	Gly	Asn	Gln	Gln	Ser	Asp	Ala	Gly	Ser	Leu	Ala	Gly	195	200	205	
Thr	Gly	Gly	Gly	Leu	Gly	Thr	Pro	Ser	Ser	Phe	Ser	Asn	Asn	Ser	Ser	210	215	220	
Val	Met	Gly	Asp	Pro	Leu	Ile	Asp	Ala	Asn	Thr	Gly	Pro	Gly	Asp	Ser	225	230	235	240
Gly	Asn	Thr	Arg	Gly	Glu	Ala	Gly	Gln	Leu	Ile	Gly	Glu	Leu	Ile	Asp	245	250	255	

- 67 -

Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val  
                   260                                  265                                  270  
 Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln  
                   275                                  280                                  285  
 Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala  
                   290                                  295                                  300  
 Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala  
                   305                                  310                                  315                                  320  
 Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg  
                   325                                  330                                  335  
 Asn Gln Ala Ala Ala  
                   340

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1026 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGCAGAGTC TCAGTCTTAA CAGCAGCTCG CTGCAAACCC CGGCAATGGC CCTTGTCTCTG	60
GTACGTCTCTG AAGCCGAGAC GACTGGCAGT ACGTCGAGCA AGGCGCTTCA GGAAGTTGTC	120
GTGAAGCTGG CCGAGGAACT GATGCGCAAT GGTCAACTCG ACGACAGCTC GCCATTGGGA	180
AAACTGTTGG CCAAGTCGAT GGCCGCAGAT GGCAAGGCGG GCGGCGGTAT TGAGGATGTC	240
ATCGCTGCGC TGGACAAGCT GATCCATGAA AAGCTCGGTG ACAACTTCGG CGCGTCTGCG	300
GACAGCGCCT CGGGTACCGG ACAGCAGGAC CTGATGACTC AGGTGCTCAA TGGCCTGGCC	360
AAGTCGATGC TCGATGATCT TCTGACCAAG CAGGATGGCG GGACAAGCTT CTCCGAAGAC	420
GATATGCCGA TGCTGAACAA GATCGCGCAG TTCATGGATG ACAATCCCGC ACAGTTTCCC	480
AAGCCGGACT CGGGCTCCTG GGTGAACGAA CTCAAGGAAG ACAACTTCCT TGATGGCGAC	540
GAAACGGCTG CGTTCCGTTT GGCACCTCGAC ATCATTGGCC AGCAACTGGG TAATCAGCAG	600
AGTGACGCTG GCAGTCTGGC AGGGACGGGT GGAGGTCTGG GCACTCCGAG CAGTTTTTCC	660
AACAACTCGT CCGTGATGGG TGATCCGCTG ATCGACGCCA ATACCGGTCC CGGTGACAGC	720
GGCAATACCC GTGGTGAAGC GGGGCAACTG ATCGGCGAGC TTATCGACCG TGGCCTGCAA	780
TCGGTATTGG CCGGTGGTGG ACTGGGCACA CCCGTAAACA CCCC GCAGAC CGGTACGTCTG	840

- 68 -

GCGAATGGCG GACAGTCCGC TCAGGATCTT GATCAGTTGC TGGGCGGCTT GCTGCTCAAG	900
GGCCTGGAGG CAACGCTCAA GGATGCCGGG CAAACAGGCA CCGACGTGCA GTCGAGCGCT	960
GCGCAAATCG CCACCTTGCT GGTCAGTACG CTGCTGCAAG GCACCCGCAA TCAGGCTGCA	1020
GCCTGA	1026

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 344 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met	Ser	Val	Gly	Asn	Ile	Gln	Ser	Pro	Ser	Asn	Leu	Pro	Gly	Leu	Gln	1	5	10	15
Asn	Leu	Asn	Leu	Asn	Thr	Asn	Thr	Asn	Ser	Gln	Gln	Ser	Gly	Gln	Ser	20	25	30	
Val	Gln	Asp	Leu	Ile	Lys	Gln	Val	Glu	Lys	Asp	Ile	Leu	Asn	Ile	Ile	35	40	45	
Ala	Ala	Leu	Val	Gln	Lys	Ala	Ala	Gln	Ser	Ala	Gly	Gly	Asn	Thr	Gly	50	55	60	
Asn	Thr	Gly	Asn	Ala	Pro	Ala	Lys	Asp	Gly	Asn	Ala	Asn	Ala	Gly	Ala	65	70	75	80
Asn	Asp	Pro	Ser	Lys	Asn	Asp	Pro	Ser	Lys	Ser	Gln	Ala	Pro	Gln	Ser	85	90	95	
Ala	Asn	Lys	Thr	Gly	Asn	Val	Asp	Asp	Ala	Asn	Asn	Gln	Asp	Pro	Met	100	105	110	
Gln	Ala	Leu	Met	Gln	Leu	Leu	Glu	Asp	Leu	Val	Lys	Leu	Leu	Lys	Ala	115	120	125	
Ala	Leu	His	Met	Gln	Gln	Pro	Gly	Gly	Asn	Asp	Lys	Gly	Asn	Gly	Val	130	135	140	
Gly	Gly	Ala	Asn	Gly	Ala	Lys	Gly	Ala	Gly	Gly	Gln	Gly	Gly	Leu	Ala	145	150	155	160
Glu	Ala	Leu	Gln	Glu	Ile	Glu	Gln	Ile	Leu	Ala	Gln	Leu	Gly	Gly	Gly	165	170	175	
Gly	Ala	Gly	Ala	Gly	Gly	Ala	Gly	Gly	Val	Gly	Gly	Ala	Gly	Gly		180	185	190	
Ala	Asp	Gly	Gly	Ser	Gly	Ala	Gly	Gly	Ala	Gly	Gly	Ala	Asn	Gly	Ala	195	200	205	

- 69 -

Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn  
 210 215 220  
 Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp  
 225 230 235 240  
 Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn  
 245 250 255  
 Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Gly Asn Gln  
 260 265 270  
 Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly  
 275 280 285  
 Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser  
 290 295 300  
 Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val  
 305 310 315 320  
 Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln  
 325 330 335  
 Gln Ser Thr Ser Thr Gln Pro Met  
 340

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1035 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGTCAGTCG GAAACATCCA GAGCCCGTCG AACCTCCCGG GTCTGCAGAA CCTGAACCTC	60
AACACCAACA CCAACAGCCA GCAATCGGGC CAGTCCGTGC AAGACCTGAT CAAGCAGGTC	120
GAGAAGGACA TCCTCAACAT CATCGCAGCC CTCGTGCAGA AGGCCGCACA GTCGGCGGGC	180
GGCAACACCG GTAACACCGG CAACGCGCCG GCGAAGGACG GCAATGCCAA CGCGGGCGCC	240
AACGACCCGA GCAAGAACGA CCCGAGCAAG AGCCAGGCTC CGCAGTCGGC CAACAAGACC	300
GGCAACGTCG ACGACGCCAA CAACCAGGAT CCGATGCAAG CGCTGATGCA GCTGCTGGAA	360
GACCTGGTGA AGCTGCTGAA GGCGGCCCTG CACATGCAGC AGCCCGGCGG CAATGACAAG	420
GGCAACGGCG TGGGCGGTGC CAACGGCGCC AAGGGTGCCG GCGGCCAGGG CGGCCTGGCC	480
GAAGCGCTGC AGGAGATCGA GCAGATCCTC GCCCAGCTCG GCGGCGGCGG TGCTGGCGCC	540
GGCGGCGCGG GTGGCGGTGT CGGCGGTGCT GGTGGCGCGG ATGGCGGCTC CGGTGCGGGT	600





## WHAT IS CLAIMED:

1. A method of producing plant seeds which impart pathogen resistance to plants grown from the seeds, said method comprising:

applying a hypersensitive response elicitor polypeptide or protein in a non-infectious form to a plant seed under conditions effective to impart pathogen resistance to a plant grown from the seeds.

2. A method according to claim 1, wherein the hypersensitive response elicitor polypeptide or protein is in isolated form.

3. A method according to claim 2, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from a pathogen selected from the group consisting of *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Phytophthora*, and mixtures thereof.

4. A method according to claim 3, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from *Erwinia chrysanthemi*.

5. A method according to claim 3, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from *Erwinia amylovora*.

6. A method according to claim 3, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from *Pseudomonas syringae*.

7. A method according to claim 3, wherein the hypersensitive response elicitor polypeptide or protein

- 72 -

corresponds to that derived from *Pseudomonas solanacearum*.

8. A method according to claim 3, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from *Xanthomonas campestris*.

9. A method according to claim 3, wherein the hypersensitive response elicitor polypeptide or protein corresponds to a *Phytophthora* species.

10. A method according to claim 2, wherein the plant is selected from the group consisting of dicots and monocots.

11. A method according to claim 10, wherein the plant is selected from the group consisting of rice, wheat, barley, rye, oats, cotton, sunflower, canola, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

12. A method according to claim 10, wherein the plant is selected from the group consisting of rose, *Saintpaulia*, petunia, *Pelargonium*, poinsettia, chrysanthemum, carnation, and zinnia.

13. A method according to claim 2, wherein the pathogen to which the plant is resistant is selected from the group consisting of viruses, bacteria, fungi, and combinations thereof.

- 73 -

14. A method according to claim 2, wherein said applying is carried out by spraying, injection, coating, dusting or immersion.

5 15. A method according to claim 2, wherein the hypersensitive response elicitor polypeptide or protein is applied to plant seeds as a composition further comprising a carrier.

10 16. A method according to claim 15, wherein the carrier is selected from the group consisting of water, aqueous solutions, slurries, and powders.

15 17. A method according to claim 15, wherein the composition contains greater than .5 nM of the hypersensitive response elicitor polypeptide or protein.

20 18. A method according to claim 15, wherein the composition further contains additives selected from the group consisting of fertilizer, insecticide, nematocide, fungicide, herbicide, and mixtures thereof.

25 19. A method according to claim 1, wherein the hypersensitive response elicitor polypeptide or protein is applied as bacteria which do not cause disease and are transformed with a gene encoding the hypersensitive response elicitor polypeptide or protein.

30 20. A method according to claim 1, wherein the hypersensitive response elicitor polypeptide or protein is applied as bacteria which cause disease in some plant species, but not in those whose seeds are subjected to said applying, and contain a gene encoding the hypersensitive response elicitor polypeptide or protein.

35

21. A method according to claim 2, wherein said applying causes infiltration of the polypeptide or protein into the plant seed.

5 22. A method according to claim 2 further comprising:

planting in soil the seeds to which the hypersensitive response elicitor protein or polypeptide has been applied and

10 propagating plants from the planted seeds.

23. A method according to claim 22 further comprising:

15 applying the hypersensitive response elicitor polypeptide or protein to the propagated plants to enhance the plant's pathogen resistance.

24. A method according to claim 2, wherein the hypersensitive response elicitor protein or polypeptide  
20 is a fungal hypersensitive response elicitor.

25. A pathogen-resistance imparting plant seed to which a non-infectious hypersensitive response elicitor polypeptide or protein has been applied.  
25

26. A pathogen-resistance imparting plant seed according to claim 25, wherein the hypersensitive response elicitor polypeptide or protein is in isolated form.  
30

27. A pathogen-resistance imparting plant seed according to claim 26, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from a pathogen selected from the group

- 75 -

consisting of *Erwinia*, *Pseudomonas*, *Xanthomonas*,  
*Phytophthora*, and mixtures thereof.

28. A pathogen-resistance imparting plant seed  
5 according to claim 27, wherein the hypersensitive  
response elicitor polypeptide or protein corresponds to  
that derived from *Erwinia chrysanthemi*.

29. A pathogen-resistance imparting plant seed  
10 according to claim 27, wherein the hypersensitive  
response elicitor polypeptide or protein corresponds to  
that derived from *Erwinia amylovora*.

30. A pathogen-resistance imparting plant seed  
15 according to claim 27, wherein the hypersensitive  
response elicitor polypeptide or protein corresponds to  
that derived from *Pseudomonas syringae*.

31. A pathogen-resistance imparting plant seed  
20 according to claim 27, wherein the hypersensitive  
response elicitor polypeptide or protein corresponds to  
that derived from *Pseudomonas solanacearum*.

32. A pathogen-resistance imparting plant seed  
25 according to claim 27, wherein the hypersensitive  
response elicitor polypeptide or protein corresponds to  
that derived from *Xanthomonas campestris*.

33. A pathogen-resistance imparting plant seed  
30 according to claim 27, wherein the hypersensitive  
response polypeptide or protein corresponds to that  
derived from a *Phytophthora* species.

34. A pathogen-resistance imparting plant seed  
35 according to claim 26, wherein the plant seed is for

plants selected from the group consisting of dicots and monocots.

5 35. A pathogen-resistance imparting plant seed according to claim 34, wherein the plant is selected from the group consisting of rice, wheat, barley, rye, oats, cotton, sunflower, canola, peanut, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion,  
10 garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

15 36. A pathogen-resistance imparting plant seed according to claim 34, wherein the plant is selected from the group consisting of rose, *Saintpaulia*, petunia, *Pelargonium*, poinsettia, chrysanthemum, carnation, and zinnia.

20 37. A pathogen-resistance imparting plant seed according to claim 27, wherein the pathogen to which the plant is resistant is selected from the group consisting of a virus, bacterium, fungus, nematode, and combinations  
25 thereof.

38. A pathogen-resistance imparting plant seed according to claim 25, wherein the plant seed cells are in contact with bacteria which do not cause disease and  
30 are transformed with a gene encoding the hypersensitive response elicitor polypeptide or protein.

39. A pathogen-resistance imparting plant seed according to claim 25, wherein the plant seed cells are  
35 in contact with bacteria which do not cause disease in

- 77 -

the plant, but do cause disease in other plant species, and contain a gene encoding the hypersensitive response elicitor polypeptide or protein.

5           40. A pathogen-resistance imparting plant seed according to claim 26, wherein the plant seed is infiltrated with the polypeptide or protein.

10           41. A method of imparting pathogen resistance to plants comprising:  
                providing a transgenic plant seed transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein;  
                planting the transgenic plant seed in  
15   soil; and  
                propagating a plant from the planted seed under conditions effective to impart pathogen resistance to the plant.

20           42. A method according to claim 39, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from a pathogen selected from the group consisting of *Erwinia*,  
                *Pseudomonas*, *Xanthomonas*, *Phytophthora*, and mixtures  
25   thereof.

                43. A method according to claim 42, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from *Erwinia*  
30   *chrysanthemi*.

                44. A method according to claim 42, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from *Erwinia*  
35   *amylovora*.

45. A method according to claim 42, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from *Pseudomonas syringae*.

46. A method according to claim 42, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from *Pseudomonas solanacearum*.

47. A method according to claim 42, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from *Xanthomonas campestris*.

48. A method according to claim 42, wherein the hypersensitive response elicitor polypeptide or protein corresponds to that derived from a *Phytophthora* species.

49. A method according to claim 41, wherein the plant is selected from the group consisting of dicots and monocots.

50. A method according to claim 49, wherein the plant is selected from the group consisting of rice, wheat, barley, rye, oats, cotton, sunflower, canola, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.



- 79 -

51. A method according to claim 49, wherein the plant is selected from the group consisting of rose, *Saintpaulia*, petunia, *Pelargonium*, poinsettia, chrysanthemum, carnation, and zinnia.

5

52. A method according to claim 41, wherein the pathogen to which the plant is resistant is selected from the group consisting of viruses, bacteria, fungi, and combinations thereof.

10

53. A method according to claim 41 further comprising:

applying the hypersensitive response elicitor polypeptide or protein to the propagated plants to enhance the plant's pathogen resistance.

15

54. A method according to claim 41, wherein the hypersensitive response elicitor protein or polypeptide is a fungal hypersensitive response elicitor.

20

55. A plant produced by the method of claim 22.

56. A plant seed from the plant produced by the method of claim 22.

25

57. A plant propagule from the plant produced by the method of claim 22.

30

58. A plant produced by the method of claim 41.

59. A plant seed from the plant produced by the method of claim 41.

35

60. A plant propagule from the plant produced  
by the method of claim 41.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/22629

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 800/200, 250; 514/2; 530/370

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/200, 250; 514/2; 530/370

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Sequence, CAS - Agriculture and Bioscience Clusters

Search terms: hypersensitive, elicit?, harpin, seed, spore, tuber, kernel, pit, pip

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 5,550,228 A (GODIARD, et al.) 27 August 1996, entire document but specifically col. 4, lines 46-59, col. 5, lines 30-41, cols. 5-6 lines 59-7.	19-20, 41-42, 44, 46, 49-50, 52, 58-60  43, 45, 47-49, 51
A	US 5,552,527 A (GODIARD et al.) 09 September 1996, entire document.	
Y	WO 94/01546 A1 ( CORNELL RESEARCH FOUNDATION, INC) 20 January 1994, entire document.	43, 45, 47-49, 51
A	WO 94/26782 A1 (CORNELL RESEARCH FOUNDATION, INC.) 24 November 1994.	

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 MARCH 1998

Date of mailing of the international search report

07 APR 1998

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/22629

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 96/39802 A1 (CCORNELL RESEARCH FOUNDATION, INC.) 19 December 1996, entire document.	1-40, 55-57
X,P	WEI, et al., Hypersensitive response induced resistance in plants, Cornell Research Foundation, Inc. 1997. AN ; 639888.	1-40, 55-57
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/22629

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A01G 1/00, A01H 1/00, 1/02, 5/00, 7/00, 9/00, 11/00, 13/00, 15/00, 17/00; A01N 37/18; A61K 35/78, 35/80, 38/00;  
C07K 1/00, 14/00, 16/00, 17/00

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